THE PHYSIOLOGICAL AND IMMUNOLOGICAL EFFECTS OF ASTAXANTHIN ON STRESSED NILE TILAPIA REARED IN A COUPLED-AQUAPONICS SYSTEM

by

Lindee T. Mason

A Thesis

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Master of Science

Department of Biology at Purdue Fort Wayne

Fort Wayne, Indiana

May 2021

THE PURDUE UNIVERSITY GRADUATE SCHOOL
STATEMENT OF COMMITTEE APPROVAL

Dr. Ahmed Mustafa, Chair
Department of Biology

Dr. Scott Bergeson
Department of Biology

Dr. Jordan Marshall
Department of Biology

Approved by:
Dr. Jordan M. Marshall

To June Fitzgerald, rest in heavenly peace, grandma.

ACKNOWLEDGMENTS

First, I would like to thank the person who has had the most significant impact on my life and college career. Dr. Ahmed Mustafa has been my mentor for nearly four years now, has helped me realize my passion for fish biology and aquaculture. This research would not have been possible without his guidance and support. Dr. Mustafa has helped me realize my true potential, and for that I will forever be grateful. Without him I am not sure where I would be today.

I would also like to thank the members of my committee, Dr. Jordan Marshall and Dr. Scott Bergeson. Dr. Marshall, thank you for teaching me everything I know about statistics and answering my millions of questions. I would also like to thank you for your guidance throughout the grad school application process and throughout grad school. When I was on the fence about going to grad school or vet school, I went to Dr. Bergeson for guidance, because of him I opted out of veterinary school. Attending grad school probably was the best thing I could have done, and for that I thank you.

I would also like to thank Kenneth Saillant and Destin Furnas for teaching me everything that I needed to know about the many different aquaculture systems that we have on campus. Without them I would not have know how to conduct my research. My two main supporters throughout this research were Aparna Biswas and Asif Mortuza, without them none of this would have been possible. The pandemic hit right at the beginning of my research, so our usual 10-men team was now a three-men team. Thank you both for all of your hard work, assistance, and guidance throughout my research. I would also like to thank Abdul Akbar, Malaika Ahmed, Rosemary Burch, Megan Airgood, Su Neyman, and Cody Davison for all of their help in the early stages of my research, without them this would not have been possible.

I would also like to thank the many people within the biology department who have made my life so much easier during these past two years. Colleen Krohn, I literally do not know what I would have done without all of your help and wisdom. Bruce Arnold, thank you for always helping me when I needed to order or purchase supplies for the lab and for helping me fix equipment that was not working properly. I would like to thank Arlis LaMaster for all of her guidance in all things safety and for making the nitrifying and formalin killed bacteria for my experiments. Carla Barrett for making sure that the facilities where we keep the fish are adequate and for helping teach me about animal care and safety. Even though our time together was short I would like to thank Dar

Bender for all of her guidance during my first year of grad school and Karen Morthorst for all of your assistance through my second year of grad school.

Finally, I would like to thank my mom, dad, siblings, and grandparents for all of their love, support, and continuous words of encouragement. Being a first-generation college student was hard, but with all of you by my side it was made a little easier. I would also like to thank my friends Paul Bojrab and Meriana Nadrous for being a constant in my life throughout college and for encouraging me to keep going.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Even though aquaculture may solve the world's need for a substantial protein source, it also has its problems, with stress being the most significant issue. Stress causes a decrease in disease resistance. This causes fish to become more susceptible to bacterial infections and viruses, causing them to become ill. Because of stress, farmers experience a significant amount of financial loss every year. To treat the effects of stress on fish, antibiotics and other chemicals are used. The use of antibiotics and other chemicals in aquaculture are damaging ecosystems, increasing antibiotic resistance, and impacting human health. Nutraceuticals, food or food additivities with medicinal properties, are an alternative to using antibiotics and other chemicals. Astaxanthin (AST), a keto-carotenoid, has been shown to have strong antioxidant, anti-diabetic, anti-cancer, and immunomodulation properties, however effects of AST on the modulation of stress in fish has not been investigated extensively. The objective of this experiment was to investigate the effect of AST on the stress response of Nile tilapia (*Oreochromis niloticus)* by measuring the blood glucose, packed cell volume, spleen-somatic index, serum lysozyme activity, macrophage phagocytic capacity and condition factor. There were three experimental groups: control, stressed (hydrocortisone supplemented diet) and stressed treated with astaxanthin (hydrocortisone and AST supplemented diet). Fish were fed the designated diets over a month and sampled at day 0 and day 28 (4 weeks). There were only significant differences between the control and stressed groups for blood glucose and spleen-somatic index. These two parameters indicate that the fish in the stressed group were stressed. There were no significant differences between the stress and AST group and the stressed and groups. These data suggests that a diet supplemented with the current amount of AST (200 mg/kg of feed) is not able to modulate the stress response of Nile tilapia. Further research will need to be conducted to determine if AST will have a therapeutic effect on the stress response.

CHAPTER 1. INTRODUCTION

The ocean, as well as many other water sources have been used as a food source for a millennium. For a while, the world's water sources were able to keep up with the demand for food, but there came a point when demand started to surpass supply. This increase in demand was fueled by the world's ever-growing population. In 1999 the world's population was 6.1 billion and increased to 7.7 billion people in 2019 [1], which is a 26% increase in just 20 years. The United Nations is predicting that the world's population will increase to 8.5 billion in 2030, 9.7 billion in 2050 and 10.9 billion in 2100 [1]. Two of the many problems that an increase in human population causes is the challenges in combating hunger and malnutrition [1]. Increase in population not only causes a food availability predicament; it can also destroy our natural resources if we let it. In 2008 it was reported that the demand in seafood caused 53% of marine habitats to be fully exploited, 28% to be overexploited, 3% to be depleted and 1% were currently recovering from depletion [2]. In order to support the demand for food, a more substantial way to produce large quantities of food, without depleting natural resources is needed.

A solution to the food problem could be aquaculture. Aquaculture is the farming of aquatic plants and animals [2]. It is a relatively ancient concept dating to 4,000 – 5,000 years ago, but it has only been considered a major industry for the last 50 – 60 years [2]. Since 1970 aquaculture has had an annual growth rate of 6.6%, this makes it the fastest animal food-producing sector [2]. In 2008 the United States was the sixth largest exporter (4.5 million dollars) and the second largest importer of fish (14.1 million dollars) and fishery products [2]. It has been shown that 90% of the seafood purchased/consumed in the US is exported from other countries [3]. To keep up with this demand and its ability to grow a large amount of food in a short period time, the aquaculture industry continues to grow.

There are three main forms of aquaculture -- intensive, semi-intensive, and extensive aquaculture [4,5]. Intensive aquaculture is the farming of fish in raceways or other tanks where the natural environment is replicated (ex. water aeration) and the fish are fed by the farmer [4,5]. Extensive aquaculture is when fish are usually kept in a pond set up where there is organic material that the fish can feed on and all water parameters are met without intervention [4,5]. Semi-intensive aquaculture is a mixture of intensive and extensive aquaculture. The fish are kept in a natural

environment, such as a pond, but feeding may be done by the farmer despite the access to the organic feed that is present in the pond [4,5].

There are a few different types of intensive aquaculture, such as recirculating aquaculture systems (RAS) and raceway (flow-through) systems [5]. RAS are a recent development that has made huge advancements in the aquaculture industry. In some climates only certain species can be farmed outside during certain months, using RAS eliminates the climate restraint on fish farming since it can be done indoors [5]. RAS are also less spacious when compared to semi-intensive and extensive operations. Taking up less space allows for an increased yield of fish with a decreased amount of space. Recirculating aquaculture systems also use less water than other aquaculture systems [6]. RAS work by recirculating the "used", wastewater back through the system. Wastewater from the fish tanks is passed into a water reservoir that holds the polluted water. This reservoir holds a media that traps fecal material and other large debris before it enters the filtering mechanism. The water is then passed through a filter (usually a sand filter) that cleans the water by trapping any leftover fecal material and debris. Water is then passed through a biological filtering system that helps break down toxic ammonia into its less toxic forms (nitrite and nitrate) [5,6]. The now cleaned water flows back into the fish tanks, and the cycle starts all over. This design also decreases the workload of fish farmers since the filtering systems do most of the cleaning, the need for manual cleaning (via vacuuming or siphoning) is diminished. Since RAS filter and detoxify the wastewater [5,6], they are also more eco-friendly than other systems commonly used in aquaculture. According to *A Guide to Recirculating Aquaculture* from the FAO, a flow-through system uses 1,712,000 liters of new water when producing 500 tons of fish/year and a low-level recirculating system uses 171,000 liters of new water when producing the same number of fish [6]. That means less water is taken from the environment and less wastewater is released into ecosystems, potentially reducing pollution.

One form of recirculating aquaculture that has been gaining popularity is aquaponics. Aquaponics is a farming method that combines the practices of hydroponics and aquaculture, allowing the farming of plants and fish within the same unit space. Aquaponics systems started to be used in the United States in the 1970s as part of the integrated agri-aquaculture systems (IAAS) [7]. The purpose of the introduction of aquaponics was to take advantage of the resources that aquaculture and plant production have in common in order to develop products that are more economically viable and to create a primary production practice that is more sustainable [7].

Aquaponics works by using the nutrients that the fish expel in their waste as nutrients for the plants. The aquaponics system used for this research contains nitrifying bacteria that would break down the ammonia into an organic nitrogen fertilizer that the plants can then take up (some aquaponics systems do not use nitrifying bacteria). Fish waste also contains nutrients that the fish do not need, so the plants are also able to uptake other nutrients such as calcium (C), phosphorus (P), magnesium (Mg), etc. via the feed that the fish are eating (Figure 1) [7,8]. Once the plants absorb the excess nutrients in the water, the clean, detoxified water is ready to be used again by the fish. Aquaponics systems that allow this return of water from the hydroponic portion of the system to the aquaculture portion is known as a coupled-aquaponics system. There are also decoupled aquaponics systems that keep the water used by the plants and by the fish separated [7]. The benefits of using coupled-aquaponics systems is that they result in higher yields of plants and fish compared with unconnected fish and plant cultivation. Using these systems allows the most efficient use of resources, feed for nutrient input for example, and allows for increased fish health [7,8].

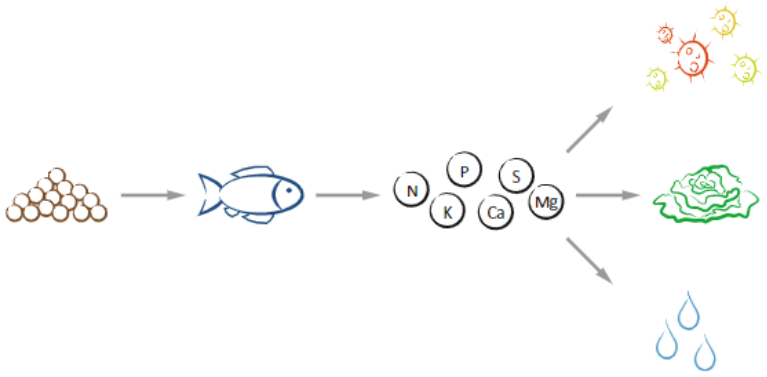

Figure 1: The process of how an aquaponics system works. The fish eat the feed, take up the required nutrients and then excrete the excess nutrients. The waste is then used as microbes and for nutrients for plants. The water is then reused in the system [7].

The use of aquaponics can also help with the increased demand of food that the ever-increasing human population has caused. Due to soil degradation, which humans may be responsible for, about 20 – 30% of the land in the world can be used for agriculture [9], but only a third of that land is arable [10]. Since the amount of land needed to grow food is declining due to soil quality, using a soilless system such as aquaponics to keep up the increasing demand of food caused by the ever-increasing human population is much needed. Since aquaponics allows the combination of edible plant and fish farming in the same unit space, the space needed to grow

these crops are minimized. These types of systems do not require arable land to grow product, therefore, again, proving them useful in food production. Along with the fact that these systems do not need soil, they also do not need any additional fertilizer for the plants to flourish [11]. For all of these reasons, aquaponics systems have been increasing in popularity across the globe.

Aquaponics, as well as any type of animal farming, has its fair share of problems. One major problem using aquaponics is animal stress. Stress can be defined as any response to a stimulus that alters homeostasis or more commonly defined as a nonspecific response of the body to any demand made upon it [12]. Stress can either be acute or chronic, depending on the type of stressor. Acute stress occurs when the body is exposed to a stressor for short periods of time, but if the stressor is persistent the response can become chronic. Acute stress has been known to be beneficial or adaptive whereas chronic stress has been known to be maladaptive [13]. Both stress responses exceed physiological tolerance limits that impairs reproductive success, growth, resistance to infectious disease, and ultimately survival [14].

Acute stress signals the release of epinephrine and norepinephrine from the adrenal medulla via the sympathetic nervous system. This short-term release of epinephrine and norepinephrine is what signals the fight or flight response and may increase immune function [13]. The body's response to chronic stress is a little bit different. When a stressor is first perceived the hypothalamus in the brain is signaled by the amygdala to release corticotrophin-releasing hormone (CRH) [13,15,16]. The presence of CRH signals the anterior pituitary gland to release adrenocorticotropic hormone (ACTH). ACTH signals the release of cortisol from the zona fasciculata layer of the adrenal cortex [13,15,16]. Cortisol (also known as the body's primary stress hormone) is a steroid hormone that is synthesized by cholesterol [15]. Cortisol has the ability to affect almost every organ system within the body by mediating the stress response and regulating the metabolism, the inflammatory response, and immune function [15]. The pathway in which cortisol is released is known as the hypothalamus-pituitary-adrenal (HPA) axis [12,13,15,16]. Fish do not have adrenal glands, so the release of cortisol in fish follows a similar pathway as in humans known as the hypothalamus-pituitary-interrenal (HPI) axis (Figure 2) [12-14,16]. There is a structure called the head kidney (or anterior kidney) in fish that mimics the functioning of the adrenal glands and the kidney in one organ. This structure contains chromaffin cells, which are responsible for the release of epinephrine and norephinephrine, mimicking the adrenal medulla [12-14], and

interrenal cells that are responsible for the release of cortisol, therefore mimicking the function of the adrenal cortex (Figure 3) [13].

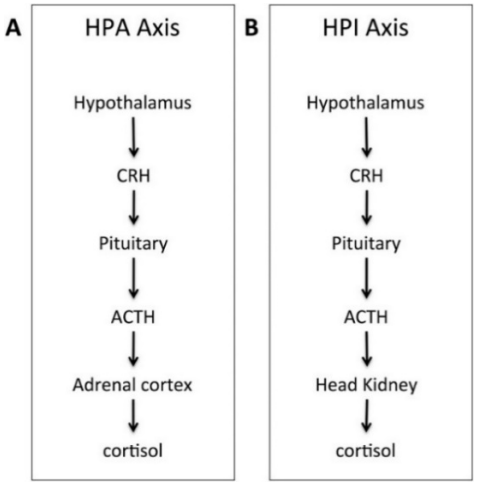

Figure 2: The hypothalamus-pituitary-adrenal (HPA) axis versus the hypothalamus-pituitary-interrenal (HPI) axis [13].

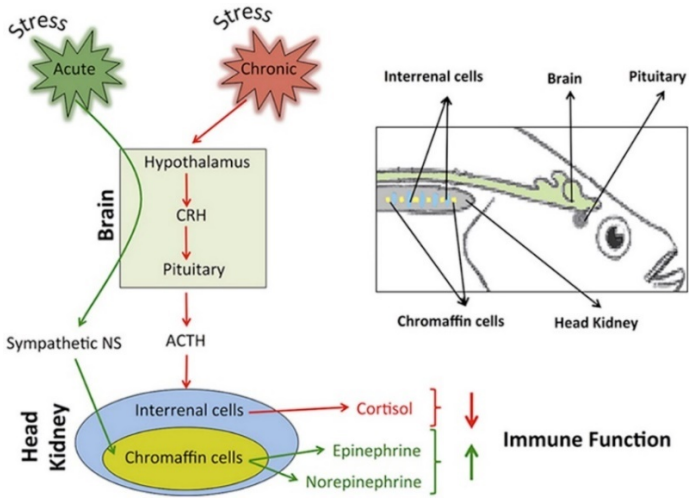

Figure 3: Acute versus chronic stress pathways in fish via the sympathetic nervous system and the hypothalamus-pituitary-interrenal (HPI) axis [13].

In aquaculture fish experience stress from being handled, transported, and vaccinated (physical stressors). However, fish can also be stressed in nature from water turbidity, pollution, predators, and temperature [14,17]. When they and confined to one space (like in aquaculture), they do not have the opportunity to move to a different area where the stressor may not be present.

Fish can also experience stress via a perceived stressor, which is when an individual perceives something as a stressor [17]. The amount of stress that an individual endures cannot be measured, but the body's response to stress can be measured in order to reflect the degree of severity of the stress experienced [12]. There are three different stages of these measurable responses: primary, secondary and tertiary [12,14]. The primary stress response mainly involves changes in the endocrine system [14]. The primary response is initiated when a stressor is perceived by the central nervous system, causing the release of hormones, such as epinephrine and norepinephrine via the sympathetic nervous system and cortisol via the HPA/HPI axis, into the blood stream [14]. The secondary stress response involves changes in the blood and tissues. Increased blood sugar concentration decreased blood-clotting time and diuresis followed by loss in blood electrolytes and osmoregulation dysfunction occur [14]. As for tissue changes, the amount of liver glycogen and interrenal vitamin C concentration will deplete, the thymus will hemorrhage and the interrenal body (kidney) may become hypertrophic [14]. The final response is the tertiary stress response. This response is characterized by whole body changes. The tertiary response causes decrease in growth, disease resistance, reproductive success, and an increased risk of death (Figure 4) [14].

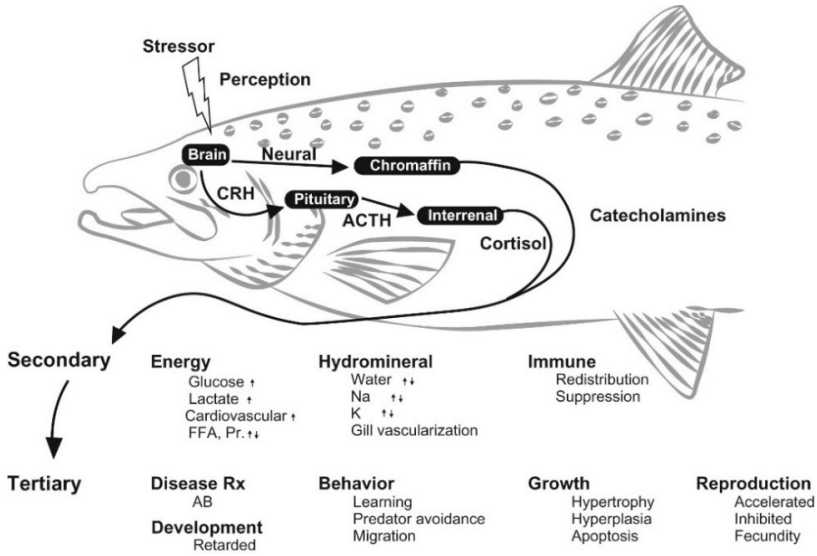

Figure 4: The primary (release of cortisol and catecholamines), secondary and tertiary stress responses. FFA, free fatty acids; P. proteins; AB, antibodies [18].

In aquaculture stress can be managed by proper husbandry and reduced handling and transport. Unfortunately, it is common practice to crowd fish in confined spaces in order to obtain

high productivity, especially in RAS [19]. As previously mentioned, stress can decrease the resistance of infectious diseases [12-14], meaning that many fish become disease ridden from poor stress management in aquaculture [21-24]. The main priority in farming is selling agricultural products in order to return a profit, but if fish are sick and unsellable the farmer loses profit. In fact, losing fish due to infectious diseases causes a multi-billion-dollar loss every year [21]. To deal with this problem, currently, farmers use antibiotics and other chemicals to help fight off diseases and to help modulate the many other consequences of stress [20-24]. Among all the chemicals that are used in all forms of agriculture, antibiotics are the most widely used to treat and prevent diseases as well as to promote animal growth [22,23,24]. When antibiotics are not fully broken down by the body, they can remain active long after being expelled from the body [22]. In some cases, excreted antibiotics have been shown to have a half-life of 300 days in aquatic environments [22]. Once the wastewater containing contaminated excrement and uneaten feed is released into nature, a myriad of problems arise.

One of the biggest problems of released antibiotics in nature is the increase in antibiotic resistance in bacteria [22-24]. Antibiotic use in aquaculture has also been shown to disrupt the natural microbial communities within aquatic ecosystems by acting as an ecological barrier [22-24]. This effects the phylogenetic structure, increases resistance, and decreases ecological function [22-24]. Antibiotics are capable of building up inside tissues within fish, so when a fish that has been treated with antibiotics is eaten by a human or other animal, they are also consuming the antibiotic(s) [22-24]. Humans who consume fish that have been treated with antibiotics could experience allergies, toxic effects, changes in intestinal microbial fauna, and may experience antibiotic resistance [22]. If humans consume fish that have been treated with chloramphenicol, an antibiotic, they can experience aplastic anemia which can lead to very dangerous bone marrow diseases [22]. Consumers can also consume antibiotics from fish that are wild caught. The uneaten food that is carried into the nature via wastewater is eaten by wild fish, the tissues of those fish are now contaminated [22. Tendencia and D de la Peña demonstrated that antibiotics from an aquaculture farm contaminated nearby water sources [25]. Wild fish that were caught from nearby water sources contained tetracycline and quinolones, the same type of antibiotics the farm was using to treat their fish [25].

Even though antibiotics cause more harm than good, they are still used frequently in aquaculture. Due to their many negative consequences, scientists are trying to find an alternative

to antibiotics; an alternative that will not ruin the environment and poison consumers. Many studies have shown that nutraceuticals may be the solution. Nutraceuticals are food or food stuff that has medical or health benefits that may help in the prevention and/or treatment of disease [26]. Nutraceuticals work by the use of secondary metabolites to help maintain homeostasis. Nutraceuticals are known to have anti-arthritic properties; help with cold, cough, sleeping disorders, digestion, cancer prevention and osteoporosis [26]. Nutraceuticals are also known to be able to control blood pressure and cholesterol, pain, depression, and diabetes [26]. There are three different categories of nutraceuticals: herbal/natural products, dietary supplements, and functional foods [26]. A nutraceutical with physiological benefits may be effective in modulating the stress response. Functional foods are foods or food additives that have physiological benefits as well as the capability of reducing the risk or chronic diseases beyond normal nutritional functions [26]. A functional food that has been increasing in popularity in both human and fish health is Astaxanthin (3,30-dihydroxy-b,b0-carotene-4,40-dione) [27].

Astaxanthin (AST) is a ketocarotenoid [28,29] (a carotenoid that has a ketone group) that is an oxidized form of β-carotene [29]. Carotenoids are pigments that are produced within plants, phytoplankton, algae, bacteria, and a few fungi species [29]. A few examples of common pigments that are produced from carotenoids is the orange color of carrots (β-carotene), the wide range of colored peppers (β-carotene, β-cryptoxanthin, lutein, zeaxanthin, etc.) [30], the reddish/orange color of salmonid flesh, and the shell color of shrimp, crabs, lobsters, and crayfish (AST) [27-29,31]. There are two different divisions of carotenoids, carotenes, and xanthophylls (includes AST) [29]. The xanthophyll group contains oxygen-containing pigments that are responsible for the yellow, orange, and red coloration of flowers, fruits, vegetables, egg yolks, feathers, shells, and flesh of many animal species [32]. AST is biosynthesized by microalgae/phytoplankton which is eaten and accumulated in zooplankton, crustaceans, and their predators, passing the pigment up the food chain. The pigment also accumulates in fish that eat the zooplankton and/or microalgae, such as salmon [28]. There are a few different organisms that are able to biosynthesize AST, they are: green algae (*Chlorococcum* sp. and *Haematococcus pluvalis*), green microalga (*Chlorella zofingiensis*), red yeast (*Xanthophyllomyces dendrorhous*), and Gram-negative bacteria (*Agrobacterium aurantiacum*) [29,31]. Of these sources, *H. pluvialis* contains the highest amount of AST [28,29,31] at more than 30g/kg [29,31]. *H. pluvialis* is a freshwater species [33] belonging to the Chlorophyta phylum [33].

Currently, AST is used in aquaculture for tissue pigmentation in order to appeal to consumers [27-29,34]. However, AST is mainly known as a strong antioxidant [27-29,31]. This is due to its ability to donate electrons and react with free radicals in order to convert them into a more stable product and to terminate free radicals [27,31]. AST has conjugated double bonds that are responsible for electron donation (Figure 5) [27,31]. Due to its chemical composition, AST is able to form isomers which are found in nature (Figure 6) [27,29,31]. There are two stereoisomers (3S,3'S) and (3R,3'R) that are more commonly seen in nature [27,29,31]. *H. pluvialis* biosynthesizes the (3S,3'S) stereoisomer of AST [27,31]. AST's position within the cell membrane also gives it stronger antioxidant capabilities than most other antioxidants (Figure 7) [27,31]. Since AST spans across the lipid bilayer cell membrane instead of just within or on the outside, like β-carotene and vitamin C, it is able scavenge free radicals on the surface of the membrane as well as on the inside [27,31]. Other than being an antioxidant, AST also been shown to have anti-lipid peroxidation, anti-inflammation, anti-diabetic, immuno-modulation, and anti-cancer properties and may also be able to prevent cardiovascular disease [27-29,31]. A study done on yellow catfish (*Pelteobagrus fulvidraco*) showed that AST was able to increase the serum total protein levels and improve the resistance to acute stress [35]. Another study done on golden pompano (*Trachinotus ovatus*) showed that AST was able to able to increase the growth and eliminate reactive oxygen species [36]. Another study showed that AST can increase the overall quality of meat in Nile tilapia (*Oreochromis niloticus*) [34]. AST was also shown to increase the metabolism, have strong antioxidant properties, anti-diabetic properties, anti-inflammatory properties, and immuno-modulation in humans [31]. Very few studies were found testing the effects of AST on modulating the stress response within fish, especially *O. niloticus*, but AST may be able to work as a functional food to alleviate some of the effects of stress. Based on its many health benefits, I decided to test AST's effect on modulating stress as a nutraceutical.

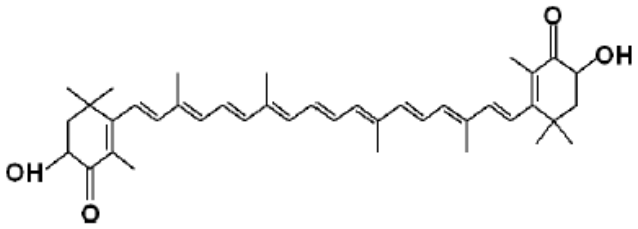

Figure 5: The chemical structure of Astaxanthin [34].

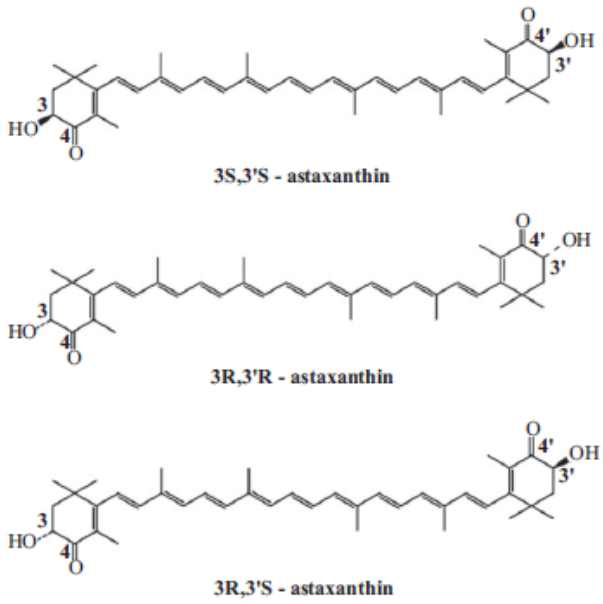

Figure 6: Three stereoisomers of Astaxanthin [29].

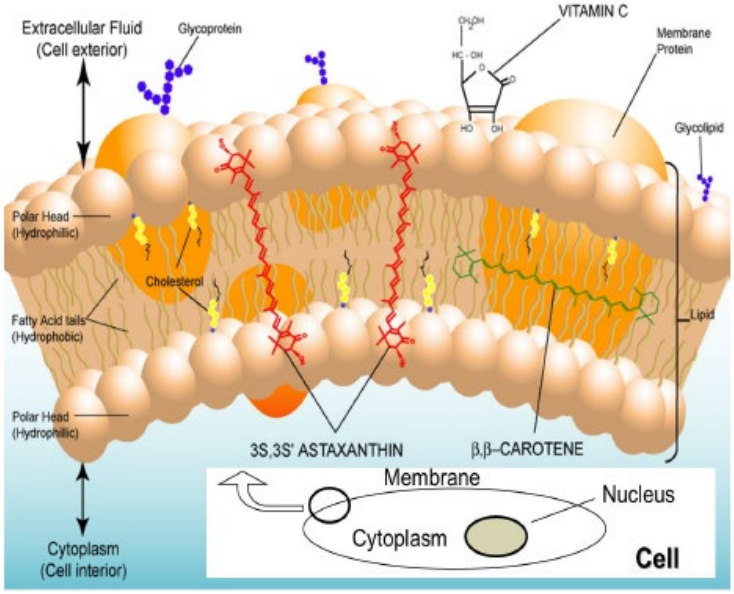

Figure 7: Position of Astaxanthin within the cell's lipid bilayer membrane [27].

This research was conducted in an aquaponics system. *O. niloticus* was chosen as my fish module since it is the most farmed species within aquaculture [11] and lettuce (*Lactuca sativa* L. [cultivar: Iceberg, variety: Batavian]) was chosen as the plant species. A portion of the fish were stressed using oral cortisol [37] and was given a feed, supplemented with AST. Due to the previously

mentioned properties of AST, the objective of this research was to determine if the addition of AST in the diet would affect the physiological response of stressed *O. niloticus*, reared in a coupled aquaponics system. Due to astaxanthin's many health benefits it is proposed that its presence will help modulate the stress response in stressed *O. niloticus.*

CHAPTER 2. MATERIALS AND METHODS

2.1 Fish Acquisition and Maintenance

Nile tilapia (*Oreochromis niloticus*) fingerlings (mean length and mass were 18.01 ± 2 cm and 113.68 ± 12.1 g, respectively) obtained from Troyer Fish Farms, Geneva, Indiana. Upon arrival, all fingerlings were placed in the coupled-aquaponics system located in the Purdue University Fort Wayne Department of Biology greenhouse. The fingerlings were kept in a 300-gallon tank (sump/water reservoir, Figure 8) for two weeks so they could acclimate to their new living conditions. After the acclimation period the fish were distributed evenly across three, 40-gallon tanks (groups 1-3, Figure 8) that are also parts of the mentioned aquaponics system. The fish were then allowed to acclimate for another two weeks in the 40-gallon tanks before experimentation.

The aquaponics system was maintained by weekly tank cleanings and 20% water changes. Water used for water changes was kept in an open container that allowed for the evaporation of chlorine. The water was allowed to sit for at least 24 hours before use in the system. To make sure all chlorine was removed and that the water is free of other heavy metals, 200 mg/ 50 gallons of Safe (Seachem Laboratories, Madison, GA), a chlorine and chloramine remover, was added to the water at regular intervals. The water chemistry was checked using an API Freshwater Water Chemistry Kit. Water chemistry was measured every day during the acclimation period and then weekly for the remainder of the experimental period. Water chemistry was kept at levels described by Ostrander [38]. The ammonia was kept at <0.25 ppm, nitrate <0.5 ppm, nitrite <40 ppm, and dissolved oxygen >7.0 mg/L [38]. The average pH and temperature were 79 ± 3 °F and 25 ± 12 %. The lighting in the greenhouse was set up so there was 12 hours of light and 12 hours of darkness to represent the day/night cycle of their natural environment. Fish were taken care off following an approved animal care protocol.

2.2 Acquisition of Astaxanthin

The astaxanthin used for this experiment was in powder form and was obtained from Prescribed For Life (Fredericksburg, TX). The powder contained 5% astaxanthin in the form of green algae (*Haematococcus pluvialis*). The composition of the powder met all FDA requirements

for use in aquaculture. The certificate of analysis provided by the company, powder composition and the FDA requirements for this product can be found in Appendix A. The powder was stored in its original packaging in a cool, dry cabinet.

2.3 Experimental Design

2.3.1 Aquaponics Setup

As previously mentioned, the study was conducted in a recirculating/coupled-aquaponics system that is located on the Purdue University Fort Wayne campus. The aquaponics system consisted of four fish tanks on the bottom and two water beds and a gravel bed on top. All the parts of the system were connected so water from the fish tanks was pumped up to the gravel bed above. Nitrifying bacteria was housed in the gravel bed to break down the toxic ammonia (via nitrogen cycle) that was excreted from fish into nitrite and nitrate. From the gravel bed the detoxified water entered the two water beds where the plants could take up the nitrite and nitrate as a source of nitrogen "fertilizer". From the two water beds the purified water flowed through pipes that lead to each separate fish tank (Figure 8).

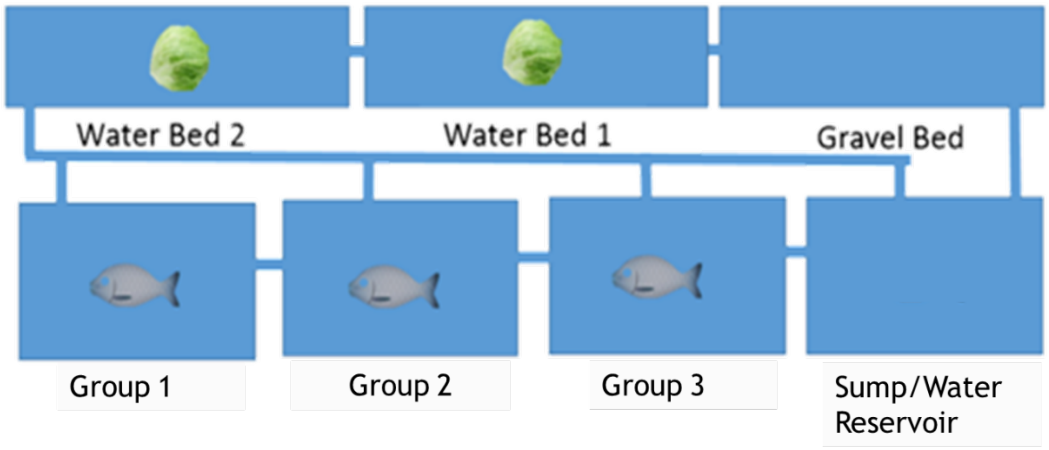

Figure 8: Aquaponics system setup.

The hydroponic portion of the aquaponics system was made up of the gravel bed and the two water beds on the top. Within the water beds 75 lettuce (*Lactuca sativa* L. [cultivar: iceberg,

variety: Batavian]) seedlings were planted. These plants were used to take up the excess nitrate and nitrite that was left over once the ammonia was broken down by the nitrifying bacteria.

The *L. sativa* seeds were purchased from Gaea's Blessing (Illinois). Seventy-five seeds were placed into 1-inch, pre-soaked rockwool starter plugs that were placed in 2-inch net pot cups. The net pot cups allow the rockwool to be placed into the floating foam board that was placed in the two water beds. The plugs were kept in shallow tubs that held an inch of water until germination and then transferred to the water beds (Figure 9). The plants were harvested when they were full of leaves that were roughly 10.16 cm in length. Only leaves that were at least four inches in length were harvested so the plants could continue to grow, allowing for more than one harvest.

Figure 9: The plastic tub contains the rockwool with seedlings that were placed into the net pots.

2.3.2 Experimental Groups

For this experiment there were three different experimental groups: Control, Stress, and Stress Astaxanthin. The Control group was housed in fish group 1, Stress in fish group 2, and Stress Astaxanthin in fish group 3 (Figure 8). Twenty-four fish were randomly divided into groups 1-3 (each group containing 8 fish). All fish were fed 1.5% of the tank body mass twice a day (a total of 3%/day). Any uneaten feed was removed from the tank after each feeding via siphoning. Before each sampling period all fish were starved for 24 hours.

2.3.3 Feed Preparation

The fish were fed with a commercial fish feed (Purina® Aquamax® Fingerling Starter 300). The commercial feed was supplemented with different ingredients (Table 1) in order to make the individual feeds needed for each experimental group. The commercial feed meets all of the nutritional requirements for omnivorous fish, like tilapia (Table 1). All of the different feeds were placed in labeled containers with lids and stored in a refrigerator until use.

For the cortisol supplemented feed, cortisol in the form of hydrocortisone powder (Acros Organics, Fair Lawn, NJ) was added to the commercial feed. According to Barton et al. (1987) 100 mg of hydrocortisone per kg of feed would keep the tilapia in a constant state of stress [37]. To add the hydrocortisone to the feed, 100 mg of the powder was dissolved in 500 mL of 200 proof ethanol. The hydrocortisone-ethanol mixture and 500 mL of fish oil was mixed with 1 kg of feed. The feed was set out overnight to dry at room temperature.

For the AST supplemented feed, 200 mg of 5% AST powder (Prescribed For Life, Fredericksburg, TX) was dissolved in 500 mL of fish oil and 500mL of 200 proof ethanol. The proper amount of the astaxanthin-fish oil mixture is added to the feed, so the 5% AST powder makes up 0.02% of the feed weight. This concentration of 10% AST powder has been shown to have therapeutic effects on golden pompano (*Trachinotus ovatus*) [36]. However, 10% AST powder was not available for purchase, so the previously mentioned study was used as a guideline. The AST mixture is mixed with a portion of the cortisol supplemented feed to make the feed for the Stress Astaxanthin group.

For the control feed, 1kg of commercial feed was mixed with 500 mL of 200 proof ethanol and 500 mL of fish oil. The feed was mixed thoroughly and laid out overnight to dry at room temperature. The addition of the ethanol and the fish oil to each feed type is to ensure the diet only differs in cortisol and astaxanthin content. Laying out the feed, unsealed, overnight allows the ethanol to evaporate before fish consumption.

Table 1: Nutritional information for the Purina® Aquamax® Fingerling Starter 300 commercial fish feed.

Content (% Feed Weight)	Control	Stress	Stress Astaxanthin
Crude Protein	50.00	50.00	50.00
Crude Fat	16.00	16.00	16.00
Crude Fiber	3.00	3.00	3.00
Calcium (Ca)	5.20	5.20	5.20
Phosphorus (P)	1.30	1.30	1.30
Sodium (Na)	0.60	0.60	0.60
Hydrocortisone	0.00	0.01[37]	0.01[37]
5% Astaxanthin	0.00	0.00	0.02[36]

2.3.4 Sampling Periods

The experiment lasted a total of four weeks and sampling occurred on day 0 and again on day 28. On day 0, three fish were sampled from each experimental group. The fish were anesthetized within two minutes of capture using 100 mg/L of tricaine methanesulfonate (Tricaine-S, MS-222) [39] (Syndel Laboratories Ltd., Nanaimo, Canada). Data collected on day 0 was to provide a baseline measurement for all experimental groups. Since the fish were not euthanized only blood parameters (blood glucose, packed cell volume, and serum lysozyme activity) were measured as well as their length and mass. Fish were allowed to recover from anesthetization in a container containing clean tank water with aeration.

The day 28 sampling was terminal so a higher dose of MS-222 (>200 mg/L) was used to euthanize the fish. This higher dose of MS-222 allows for rapid immobilization of the fish, allowing the level of cortisol to remain the same despite being handled [40] . These fish were kept in the MS-222 until opercular movement stopped, before sample collection. On day 28 all fish were euthanized, and blood parameters were measured along with the spleen-somatic index and the macrophage phagocytic capacity. Mass and length were also measured on day 28 and were used to determine Fulton's condition factor.

2.4 Experimental Parameters

All blood collected in this study was collected using heparinized 1 ml BD syringes that were affixed with 25 G needles (Becton, Dickinson and Company, Franklin Lakes, NJ). Blood was drawn from the caudal vein located near the tail on the ventral side of the fish. Once the blood was collected it was placed in a sterilized Eppendorf tube and placed on ice until needed for further analysis. For fish that were anesthetized only 1 mL of blood was collected to prevent death due to blood loss.

All tools needed for dissection (scissors, forceps, scoopulas, and scalpels, etc.), Eppendorf tubes, tools needed for interrenal cell isolation, and various sizes of tips for pipets were all autoclaved before use. The following parameters were used to determine the effect of AST on modulating the stress response.

2.4.1 Blood Glucose

Immediately after the blood was collected from each fish and before it was emptied into an Eppendorf tube, a drop of blood from the syringe was used to measure the concentration of glucose in the blood. A FreeStyle Freedom Lite glucometer (Abbott Laboratories, Chicago, IL) and FreeStyle Freedom Lite glucose test strips (Abbott Laboratories, Chicago, IL) were used to measure the blood glucose using the manufacturer's defined protocol. This method used for measuring blood glucose has been validated by Wedemeyer et al. [14] and also been used by Gensic et al. [41].

2.4.2 Packed Cell Volume

A small portion of blood from each fish, collected in the Eppendorf tubes, was used to measure the packed cell volume. Blood was transferred into Fisherbrand® Blue-Tip Plain Micro-Hematocrit Capillary Tubes (Fisher Scientific, Pittsburgh, PA) via capillary action. Each tube was filled 2/3-3/4 full and the end that was not used to draw up the blood was capped using Surgipath Critocaps (Leica Biosystems Richmond Inc., Richmond, Ill). The sealed capillary tubes were spun in a micro-hematocrit centrifuge at 10,000 RPMs for 5 minutes. Once the plasma was separated, the packed cell volume was measured using a Micro-Hematocrit Capillary Tube Reader (Leica

Biosystems Richmond Inc., Richmond, IL). This method has been established and used in previous research done by Wedemeyer et al. [14].

2.4.3 Spleen-Somatic Index

After blood collection, each fish, on day 28, was dissected and their spleen was removed. Immediately upon removal, the spleen was weighed using an analytical balance. The mass of each fish's spleen was recorded to the ten-thousandths of a gram. In order to calculate the spleen-somatic index, the somatic (body) weight was also needed. After euthanization, each fish was weighed using the following formula [42], the spleen-somatic index (SSI) was calculated:

$$SSI = \frac{spleen\ mass\ (g)}{body\ mass\ (g)} * 100$$

2.4.4 Serum Lysozyme Activity

With the left-over blood in the Eppendorf tube after measuring the blood glucose and the packed cell volume, the serum lysozyme activity was measured. The remaining blood in the Eppendorf tube was centrifuged for 10 minutes at 5000 RPMs. After centrifugation, the serum was removed and placed in a clean, sterile Eppendorf tube. In order to perform the lysozyme assay, a suspension of *Micrococcus lysodeikticus* at a concentration of 0.2 mg/mL in 0.05M (pH = 6.2) sodium phosphate buffer solution was made. The *Micrococcus lysodeikticus* suspension was vortexed before 1 mL of the suspension was placed into another clean, sterile Eppendorf tube. 50 µL of the serum was added to the Eppendorf tube containing the *Micrococcus lysodeikticus* suspension and then vortexed. This mixture was transferred to a clean cuvette and placed into a Spectronic 601 spectrophotometer (Milton Roy Company). The spectrophotometer was calibrated using pure sodium phosphate buffer. The absorbance (abs) at 530 nm was taken at 1 minute after mixing and again at 5 minutes after mixing for each sample. The following formula [43] was used to calculate the lysozyme activity (LA):

$$LA = \frac{Absorbance\ (Final) - Absorbance\ (Intial)}{Total\ Time\ Elapsed\ (minutes)}$$

2.4.5 Macrophage Phagocytic Capacity

After the spleen was removed on day 28, the head kidney was also removed. The head kidney was removed, using sterilized tools, and placed into a 15 mL centrifuge tube that contained 2 ml of Leibovitz's L-15 Medium (L-15) (Sigma, At. Louis, MO). Once the sample was deposited, it was placed on ice until needed. The contents of the centrifuge tube were then poured through a sterile metal sieve (80 mesh/190μm) and macerated using a sterile glass plunger. They were then returned to the same 15 mL centrifuge tube and centrifuged for 10 minutes at 1000 RPMs. The interrenal cells containing macrophages formed a pellet. The supernatant was carefully discarded as to not disrupt the pellet. Another 2 mL of L-15 medium was added to the centrifuge tube and vortexed until thoroughly mixed and centrifuged again for 10 minutes at 1000 RPMs. After spinning, the supernatant was discarded, and the cells were re-suspended in l mL of L-15.

To perform the macrophage assay, 50 μL of the cells re-suspended in l mL of L-15 were placed in both wells of the etched side of a double-etched microscope slide. Each sample was vortexed before placing on slides. The cells were kept moist by placing paper towels that were soaked with phosphate-buffered saline (PBS) at the bottom of the incubation tray; the cells were incubated at 25°C for 90 minutes. After incubation, 50 μL of formalin-killed *Basillus megabacterium* was added to the cells in each well. The cells were then incubated using the same method as before, but for 2 hours this time. After the final incubation, the slides were carefully washed using PBS. To fix the cells to the slides, the slides were dipped into 100% methanol for 1 minute and then stained. To stain the cells, the slides were then dipped in Wright-Giemsa stain for 1 minute. The stain was then washed from the slide, carefully, with PBS and then set out to air dry.

The number of macrophages on each slide were then counted under oil immersion (100x). 50 cells were randomly counted from both wells (100 cells/slide). Macrophages were considered positive if they had engulfed 5 or more *B. megabacterium* spores. If they engulfed less than 5 then they were considered negative. The following formula [44] was used to determine the macrophage phagocytic capacity (MPC):

$$MPC = \frac{Number\ of\ Positive\ Macrophages}{Total\ Number\ of\ Cells\ Counted} * 100$$

2.4.6 Fulton's Condition Factor

After each fish was weighed the maximum total length was also recorded (Figure 10). The weight and length of each fish on day 28 was used to calculate Fulton's condition factor (K). The following formula was used to calculate the condition factor [45]:

$$K = \frac{Mass\ (g) * 100}{((Length\ (cm))^3}$$

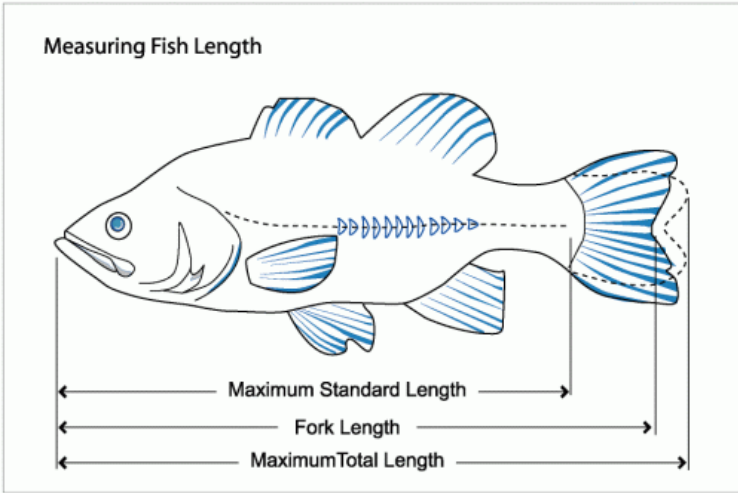

Figure 10: The maximum total length was measured to calculate K. (http://www.fsl.orst.edu/geowater/FX3/help/9_Fish_Performance/Measures_of_Fish_Length.htm).

2.4.7 Plant Weight

The *L. sativa* leaves were harvested as needed throughout the experiment. When the leaves where large in size (Figure 11; Figure 12), the outer leaves were snipped off an inch from the base (Figure 12) and the wet weight was taken. The inner leaves of each plant were kept intact so the plants were able to continue growing. There is no statistical comparison done for the plants as all the plant beds are connected to all fish groups (Figure 8).

Figure 11: *L. sativa* plants in the aquaponics system in Purdue Fort Wayne's greenhouse. Once the plants were full like the image shows, the plants were harvested.

Figure 12: Harvested *L. sativa* leaves.

2.5 Graphical and Statistical Data Analysis

All data collected from this experiment were analyzed using R (version 3.6.1). One-way analysis of variance (ANOVA) was used to compare the measured characteristics for data that were sampled for Control, Stress, and Stress Astaxanthin fish. Tukey's HSD was used as a post-hoc test to identify differences between experimental groups.

CHAPTER 3. RESULTS

3.1 Blood Glucose

For blood glucose there was a significant difference between the treatments ($F = 11.09$, $df = 2,6$, $P = 0.010$; Figure 13). The Stress group had the greatest blood glucose value, which was significantly different from the Control group. The Stress w/ AST group was not significantly different from the other two experimental groups. with a mean of 78 ± 3 mg/dL (Figure 13). The Stress w/ AST group had the second highest blood glucose with a mean of 51.5 ± 0.5 mg/dL. The Control group had the lowest blood glucose concentration with a mean of 39.5 ± 2.5 mg/dL. Tukey's test showed there was only a significant difference ($P = 0.008$) between the control and stressed groups (Figure 13). The difference between the Control and Stress Astaxanthin (Stress w/AST) groups and the difference between the Stress and Stress Astaxanthin (Stress w/AST) groups were not significantly different ($P = 0.099$; $P = 0.154$) at day 28 (Figure 13). On day 0 the mean blood glucose for Control, Stress and Stress w/ AST was 38 ± 2 mg/dL, 27.67 ± 2.2 mg/dL, and 33 ± 2.5 mg/dL, respectively. Over the span of four weeks the mean blood glucose level increased for each experimental group.

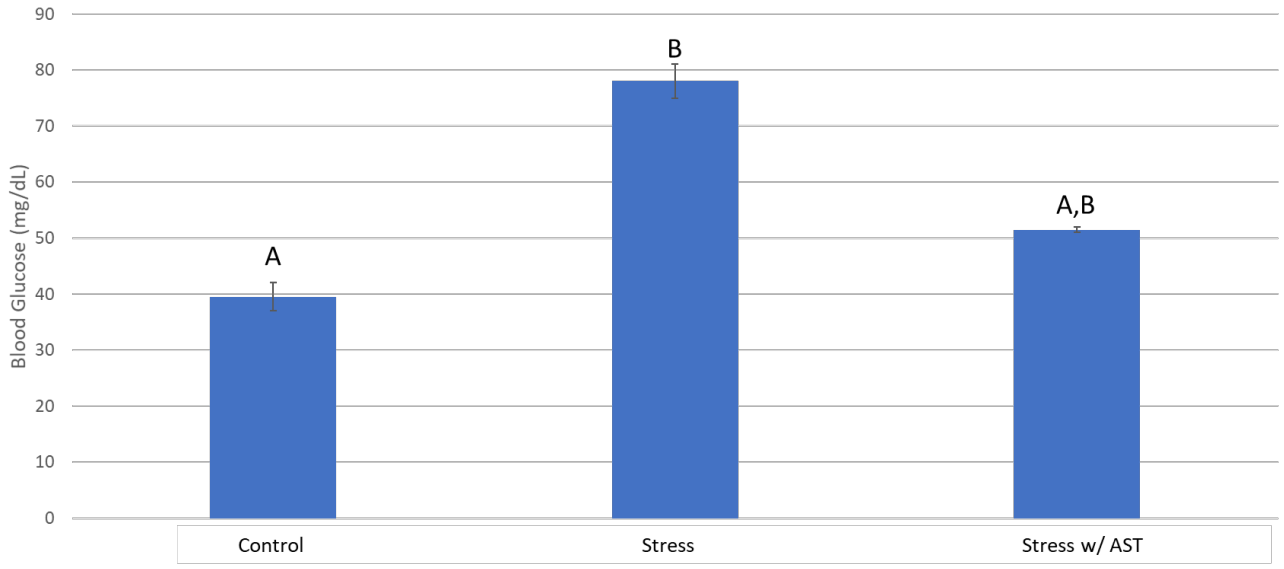

Figure 13: Mean blood glucose (mg/dL ± SE) for all experimental groups on day 28 (week 4, terminal sampling). Bars with different letters were significantly different ($P < 0.05$).

3.2 Packed Cell Volume

Packed cell volume was not different between the three experimental groups (F = 1.32, df = 2,6, P = 0.336; Figure 14). Similar to blood glucose, the Stress group had the highest packed cell volume with a mean of 33 ± 1% (Figure 14). The Stress w/ AST group had the second highest packed cell volume with a mean of 32.66 ± 1.45%. The Control group had the lowest packed cell volume with a mean of 29.5 ± 0.5%. However, the differences between the packed cell volume for the Control, Stress, and Stress w/ AST groups were not significantly different (F = 1.32, df = 2,6, P = 0.336) at day 28 (Figure 14). On day 0 the packed cell volume for Control, Stress, and Stress w/ AST was 29.33 ± 1.8 %, 36.6 ± 0.7 %, and 29 ± 0.6 %, respectively. The day 0 values are similar to those collected on day 28.

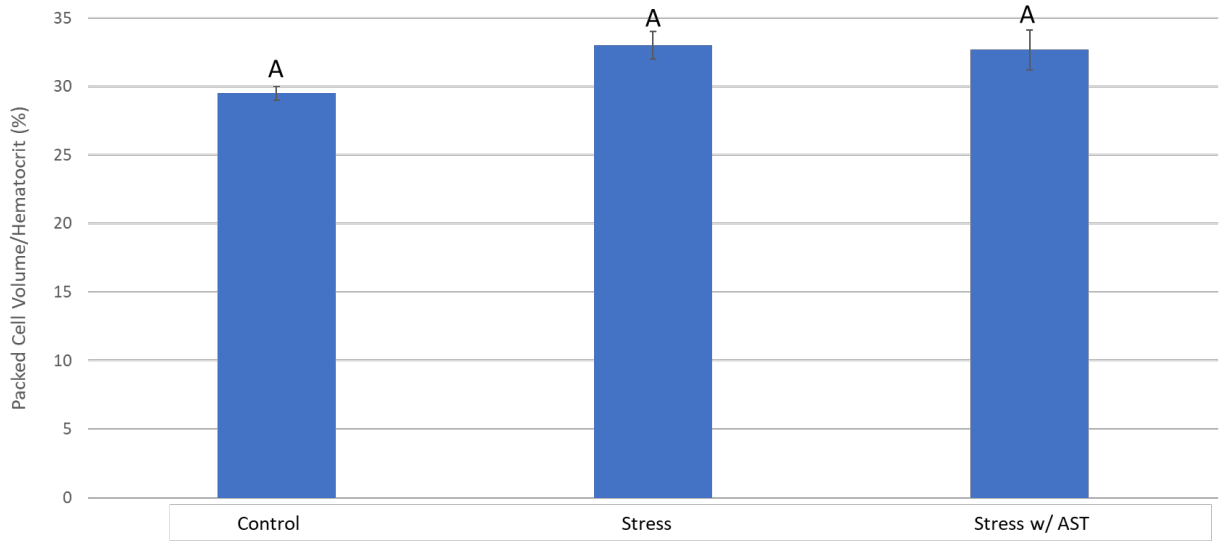

Figure 14: Mean packed cell volume/hematocrit (% ± SE) for all experimental groups for day 28 (week 4, terminal sampling). Bars with different letters are significantly different (P < 0.05). There was no significant difference between the experimental groups.

3.3 Spleen-Somatic Index

Spleen-somatic index was calculated as a ratio between spleen mass and body mass. Patterns for the spleen-somatic index were inverse to the blood glucose values. The Stress group had the lowest spleen-somatic index value, which was significantly different from the Control group (F = 5.87, df = 2,6, P = 0.039; Figure 15). The Stress w/ AST group was not significantly different from the other two experimental groups. The control group had the highest spleen-somatic index with a

mean of 0.50 ± 0.08 (Figure 15). Stress w/AST had the second highest spleen-somatic index with a mean of 0.34 ± 0.008. The Stress group had the lowest spleen-somatic index with a mean of 0.145 ± 0.03. There was a significant difference between the Control and Stress groups index (P = 0.033) at day 28 (Figure 15). There were no significant differences in the spleen-somatic index between the Control and Stress w/ AST groups (P = 0.386) and between the Stress and Stress w/ AST groups (P = 0.197) at day 28 (Figure 15).

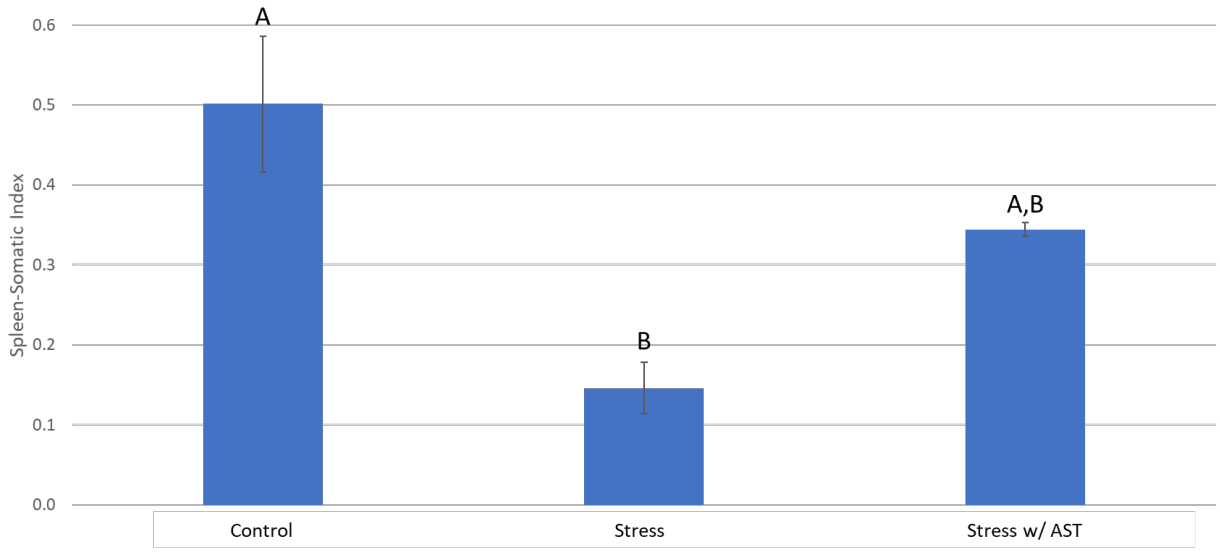

Figure 15: Mean spleen-somatic index ± SE for all experimental groups for day 28 (week 4, terminal sampling). Bars with different letters are significantly different (P < 0.05).

3.4 Serum Lysozyme Activity

Similar to packed cell volume, the serum lysozyme activity values were not significantly different between the three experimental groups (F = 1.68, df = 2,6, P = 0.263; Figure 16). The Stress group had the highest lysozyme activity with a mean of 0.011 ± 0.002 abs/minute (Figure 16). The Control group had the second highest lysozyme activity with a mean of 0.010 ± 0.001 abs/minute. The Stress w/AST group had the lowest lysozyme activity with a mean of 0.0006 ± 0.343 abs/minute. On day 0 the mean serum lysozyme activity for Control, Stress and Stress w/ AST was 0.011 ± 0.001 abs/minute, 0.011 ± 0.002 abs/minute, and 0.007 ± 0.001 abs/minute, respectively. The day 0 values are similar to those collected on day 28.

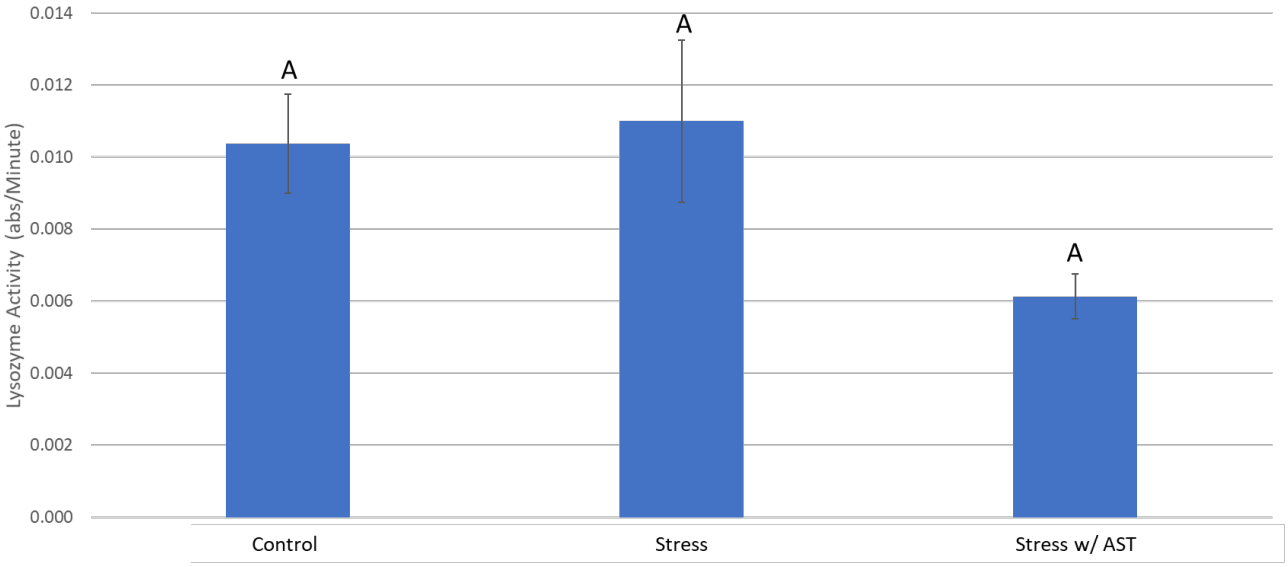

Figure 16: Mean lysozyme activity (abs/minute ± SE) for all experimental groups for day 28 (week 4, terminal sampling). Bars with different letters are significantly different ($P < 0.05$). There was no significant difference between the experimental groups.

3.5 Macrophage Phagocytic Capacity

Macrophage phagocytic capacity was calculated as a ratio between numbers of macrophage and total cells in samples. There was no significant difference between the experimental groups for macrophage phagocytic capacity ($F = 1.22$, $df = 2,6$, $P = 0.358$; Figure 17). The Stress group had the highest macrophage phagocytic capacity with a mean of 56.5 ± 5.5% (Figure 17). The Stress w/ AST group had the second highest macrophage phagocytic capacity with a mean of 28 ± 5%. The Control group had the lowest packed cell volume with a mean of 59.5 ± 20.5%.

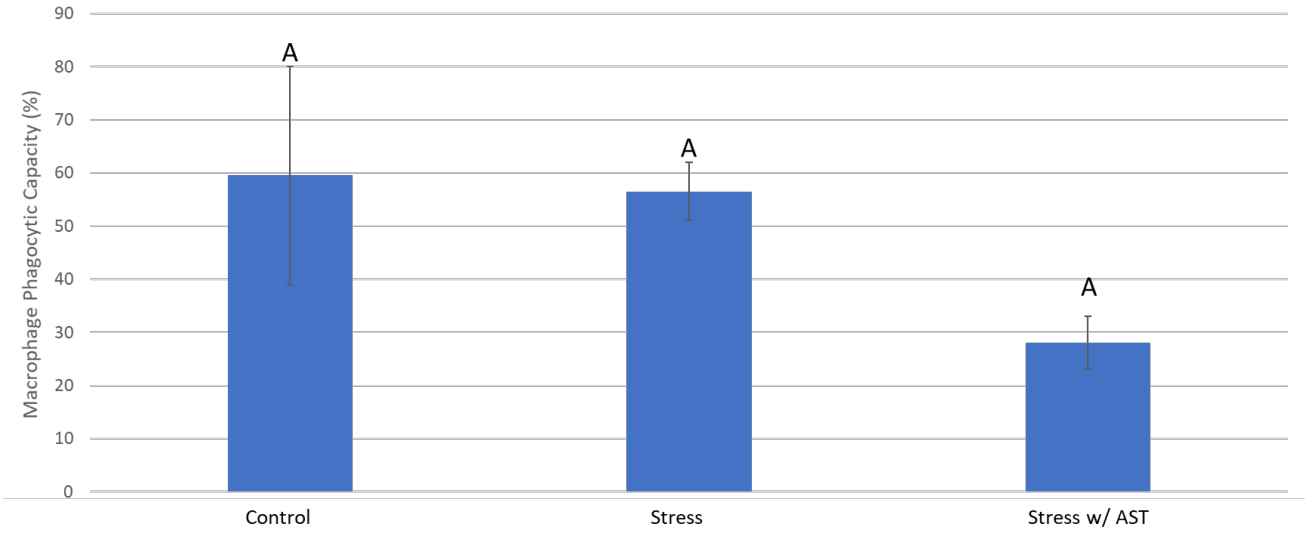

Figure 17: Mean macrophage phagocytic capacity (% ± SE) for all experimental groups for day 28 (week 4, terminal sampling). Bars with different letters are significantly different ($P < 0.05$). There was no significant difference between the experimental groups.

3.6 Fulton's Condition Factor

There was no significant difference between the experimental groups for Fulton's condition factor ($F = 2.52$, $df = 2,6$, $P = 0.161$; Figure 18). The Control group had the highest condition factor with a mean of 1.97 ± 0.13 (Figure 18). The Stress group had the second highest condition factor with a mean of 1.79 ± 0.033. The Stress w/AST group had the lowest condition factor with a mean of 1.72 ± 0.01.

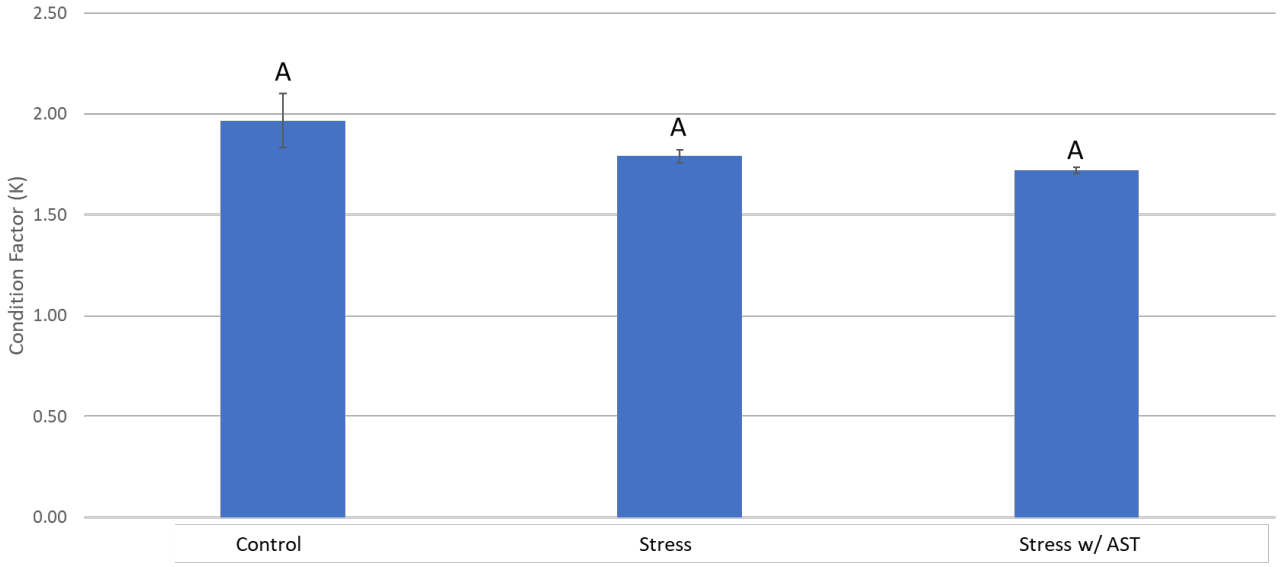

Figure 18: Mean condition Factor ± SE for all experimental groups for day 28 (week 4, terminal sampling). Bars with different letters are significantly different. There was no significant difference ($P < 0.05$) between the experimental groups.

3.7 Plant Weight

The *L. sativa* leaves were harvested twice during the experimental period. They were first harvested on May 3rd, 2020, this is when the leaves were large in size (approximately 10.16 cm in length). The plants were again harvested on May 17th, 2020, this was the final harvest for the experiment. The first harvest yielded 970 g of *L. sativa* and the second harvest yielded 376 g of lettuce for a total of 1,346 g of lettuce produced during the whole experiment. More leaves were harvested during the first harvest than in the second harvest, but harvestable leaf size remained constant.

CHAPTER 4. DISCUSSION

The purpose of this study was to determine whether an astaxanthin (AST) supplemented diet would help modulate the stress response in Nile tilapia (*Oreochromis niloticus*) by improving the physiological and immunological responses to stress. The concentration of glucose in the blood, packed cell volume, spleen somatic index, lysozyme activity, macrophage phagocytic capacity and Fulton's condition factor were used to measure the physiological and immunological responses of stress. Based on the results, AST seems to have no physiological or immunological effects on stressed *O. niloticus*. To induce the stress response, the fish feed was supplemented with hydrocortisone. This is because tilapia seem to be more resilient to common stressors that would normally stress fish out, such as handling, crowding, salinity, etc. [46-49]. Hydrocortisone was given orally to stimulate the stress response. Hydrocortisone is a synthetic version of cortisol [50]. When the diet is supplemented with hydrocortisone, the pathway that stimulates the release of cortisol (HPI axis) is inhibited, the interrenal cells do not need to release cortisol, this is because this response is controlled by negative feedback. Research has shown that this method of stressing tilapia is successful [37,51-53], so only measures of the secondary and tertiary responses were used to determine the effects of AST on the stress response.

Blood glucose is one of the most commonly used secondary stress response parameters to signify that an individual is experiencing stress [14]. Glucose is the body's main source of energy and is an essential substrate for cell metabolism [54]. As the main energy source, glucose plays a very important role in the acute stress response as it provides the body with a surge of energy that is needed for the activation of the sympathetic nervous system ("fight or flight") [54]. However, when stress goes from acute to chronic the stress response becomes maladaptive [14], causing glycogen from the liver and muscle to be depleted [14]. This is because the presence of cortisol in the blood stimulates liver glucogenesis [12,14,18] and suppresses peripheral sugar uptake [14], therefore increasing the amount of glucose in the blood, which is a sign of stress [14].

In a study done by Lim et al. [46] they found that stressed *O. niloticus* mean blood glucose level increased from 40 mg/dL to 76.17 mg/dL after six weeks [46]. Hrubec et al.[55] kept *Oreochromis* hybrids in low-density and high-density and measured the blood glucose level [55]. The low-density kept fish represent the normal basal level of glucose of the fish, which was 39 to 96 mg/dL [55]. They found that the range of blood glucose for the high-density kept fish was 30 to

69 mg/dL [55]. As previously mentioned, crowding fish can cause them stress, so when kept in high-density situations the fish will become stressed, so the range found in the density study represents a range for stressed individuals. Unlike the study done by Lim et al. [46], the fish in the Hrubec's study were chronically stressed, therefore a decrease in blood glucose level was observed [55]. The level of blood glucose for the Stress group in my study was outside of the high-density range, possibly meaning that the stressed fish were indeed stressed. The Stress Astaxanthin group had a mean blood glucose level that was lower than that of the Stress group, but this difference is not statistically significant from the Control or Stress groups. While AST did not seem to have a statistically significant effect on blood glucose, there is a potential trend. The Control group had the lowest blood glucose value, which was to be expected since the fish were not stressed, and proper husbandry methods were followed. The Stress group had the highest glucose level because of the consequences of stress and the Stress Astaxanthin group had the second highest blood glucose level maybe due to the properties of AST, but it cannot be said for sure since there was no significant difference between the Stress Astaxanthin group and the Control and Stress group.

I was expecting the Stress Astaxanthin group to have a lower blood glucose level due to AST's proposed anti-diabetic activity. AST has been shown to reduce the amount of oxidative stress that is caused due to hyperglycemia, or high blood sugar, therefore decreasing blood glucose [31]. AST is also able to increase insulin sensitivity by activating the hepatic IRS-PI3K-Akt signaling pathway [56] that is responsible for gluconeogenesis, glucose synthesis and glucose uptake [57]. Due to this mechanism, AST was shown to decrease the blood glucose for mice that were fed with a high-fructose and high-fat diet [56].

Since little research has been done in regard to the effects of AST on the stress response, finding research that pertains to fish was challenging. Contrary to my study, using yellow catfish (*Pelteobagrus fulvidraco*) found that AST was able to decrease the blood glucose 12 and 24 hours after the initiation of stress via crowding when the diet was supplemented with 8% AST at 80 mg/kg (feed weight) [35]. I used a lower concentration of AST (5%), but supplemented the feed with 200 mg/kg, based on this information, perhaps a lesser dose at a higher concentration may be more effective in modulating stress. They also tested AST's effect on acute stress where my study tested the effect on chronic stress. Another study done looked at the effect of AST on blood glucose levels of Pacific white shrimp (*Litopeanaeus vannamei*) that were exposed to salinity stress [58]. The shrimp were fed diets with 0, 40, 80, and 150 mg of AST per kg of feed. They found that the

80 mg/kg diet was able to increase the blood glucose levels [58]. Even though shrimp are not fish, or even vertebrates, the effect AST has on them is still important since the global market for farmed shrimp is growing faster than any other farmed species [59].

Packed cell volume (PCV), or hematocrit, is the percentage of red blood cells in the blood. The percentage of red blood cells, or erythrocytes, is another good secondary stress response parameter that is used to signify that an individual is being stressed [14,60]. The same density study that measured the differences in blood glucose also measured the packed cell volume for fish kept in high-density [55]. Hrubec et al.[55] found that high-density kept fish had a packed cell volume that ranged from 27 to 37% [55]. In my study the packed cell volume for each experimental group was within this range, but it cannot be determined if stress nor AST had an effect on PCV. Erythrocytes are responsible for the delivery of oxygen throughout the body, low levels of hematocrit (hemodilution) can cause anemic conditions [14]. High levels of erythrocytes in the blood (hemoconcentration) can be due to gill damage, dehydration, or stress polycythemia [14]. In order for the body to do work it needs to produce adenosine triphosphate (ATP), this is done through cellular respiration ($C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O + ATP$) [61]. Since the body needs an increased amount of energy to keep up with the consequences of stress, the body needs to increase ATP production. Since oxygen plays a major role in the production of ATP, the body needs to produce more red blood cells in order to keep up with the demand of oxygen (erythropoiesis), therefore causing a higher packed cell volume when stressed.

There is little research done on AST's impact on PCV, but the research that was found all stated that an AST supplemented diet was able to decrease the PCV. Cao and Wang [62] infected broiler chickens with a toxin to test if AST had an effect on the hematological parameters [62]. They found that AST was able to significantly reduce the PCV of infected chickens [62]. Healthy rainbow trout (*Oncorhynchus mykiss*) fed a diet supplemented with AST had a lower PCV [63]. The research did not hint at why AST was able to lower the PCV in both the chickens and the fish, but could it be correlated to the fact that AST is able to reduce the amount of blood glucose? If AST is able to decrease the amount of energy the body needs in order to cope with the consequences of stress, less ATP will be needed, so ultimately the rate of cellular respiration will decrease. In theory, this should decrease the amount of oxygen the body needs, therefore decreasing the PCV. This could be indicated by the observance of the same trend that was seen in the blood glucose result. Similar to the glucose, the Stress group had the highest PCV, Stress Astaxanthin group had the second

highest PCV and Control had the lowest, but unlike blood glucose none of these differences in PCV were significant.

The spleen is a major lymphoid organ in fish that plays a role in immunity by clearing blood-borne antigens and other immune complexes [64-66], as well as forming/making blood (hematopoiesis) [66,67], storing blood [63,73], destroying erythrocytes, releasing hemoglobin, and filtering and purifying blood [65]. Due to all of its immunological roles, the spleen can be used as an indicator of fish health [64,65]. Since stress causes a shift in the number of red blood cells in the blood (PCV) and interferes with the immune system [12-14], the spleen should be impacted due to the consequences of stress. Previous research has shown that the SSI decreases when fish are exposed to acute stressors [66]. This reduction in spleen size is due to the spleen contracting to expel blood and immune cells to compensate for the consequences of stress [66]. This initial decrease in spleen size is adaptive because it is helping the body overcome the effects of stress, but once the stressor becomes chronic or maladaptive, the decrease in spleen size becomes detrimental to fish health. Due to this response the Stress group should have the lowest SSI. The results did show that the Stress group had the lowest SSI when compared to the control, meaning stress had an effect on the SSI in this study and that they fish were stressed.

In a study done on Amur sturgeon (*Acipenser schrenckii*), there was a significant decrease in the SSI after exposure to hypoxic conditions [64]. Stressed Eurasian perch (*Perca fluviatilis*), also had a significantly reduced [68]. Additionally, hypoxia was able to decrease the spleen somatic index in *O. mykiss* [69]. There is very little research about the effect AST has on the spleen, but it has been shown that when mice were fed high doses of AST that there was a high concentration of AST in the spleen. This suggests that the spleen is the main site of AST accumulation [70]. Within the spleen AST is able to induce proliferation of splenic lymphocytes [70]. Accumulation of AST and lymphocytes in the spleen causes an increase in spleen weight [70]. Supplementation of AST into the diet of healthy *O. mykiss* had no significant effect on SSI when compared to the control [63]. AST also has the ability to increase the amount of T and B cells produced [31], but the exact mechanism on how AST is able to do this is not known thus far. With the results from these studies, we expected the Stress Astaxanthin group to have a significantly higher SSI than the Stress group , this was not the case. The Stress Astaxanthin had a higher SSI than the Stress group, but lower than the control. This could mean that maybe AST was trying to over the effect of stress on

spleen weight, but there was no significant difference between Stress Astaxanthin and Control as well as between Stress Astaxanthin and Stress.

The immunological parameters that were measured are the serum lysozyme activity and the macrophage phagocytic capacity. Since AST has been shown to modulate the immune system [27-29,31], AST supplementation was expected to improve the lysozyme activity and the macrophage phagocytic capacity. Neither parameter showed any significant differences between the three groups, however the same trend as blood glucose, PCV, and SSI was observed. Lysozyme is an enzyme that plays an important role in innate immunity [71-76]. Innate immunity is the first line of defense when the body is infected by a foreign pathogen. This immune response is a nonspecific defense, unlike the adaptive immune response, that uses physical barriers, such as skin and mucous to defend the body [75,77]. Mammals and other higher vertebrates rely on both innate and adaptive immunity to defend against pathogens, but fish mainly rely on innate immunity [75]. Since fish live in water that is rich in many different pathogens, they must have a strong innate protection [75]. Fish skin is covered in a thick mucus that contains many different components such as lysozyme, complement and interferon that help fish protect themselves against pathogens [75].

One of the major functions of lysozyme is to provide protection against microbial infection. Lysozyme destroys Gram-positive bacteria by splitting the β-(1,4) linkages between N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) in the cell wall of Gram-positive bacteria [71-76]. Lysozyme also has the ability to prevent infection from Gram-negative bacteria, but not directly like it does with Gram-positive bacteria. Lysozyme destroys Gram-negative bacteria after complementation and other enzymes have destroyed the outer cell wall layer, this exposes the inner cell wall that can be destroyed by lysozyme [72,73,75,76]. In fish, lysozyme is located in areas where the chance of bacterial infection is high, such as the gills, skin, gastro-intestinal tract, and eggs, however each different species of fish can have lysozyme in different areas and in different concentrations [75,76]. In tilapia (*Oreochromis mossambicus*) lysozyme is found mainly in the gills, serum, and liver [75], but it has been shown that *O. niloticus* has the highest amount of lysozyme in their serum [71].

Since stress can cause a decrease in immunity and resistance to diseases (bacterial infections) [12-14], lysozyme activity will be affected in stressed individuals [71,78]. It is also important to note that fluctuations in lysozyme activity during stress could be dependent on the intensity and duration of stress [71]. During acute stress lysozyme activity increases due to the

activation of the "fight or flight" response, the body is getting ready to fight [71,78]. But once stress becomes maladaptive or chronic, lysozyme activity is decreased [71,78] due to the body's lack of immunomodulation. Studies done on sheatfish (*Silurus glanis)* and *O. niloticus* showed that lysozyme activity is decreased due to chronic stress [71,78].

AST has been shown to increase resistance to bacterial infectious diseases [79]. AST also has an inhibitory effect on *Heliobacter pyl*ori growth in infected mice [31,76]. Supplementation of AST has also been shown to increase antibody production and decrease the humoral immune response [31]. While the adaptive immune response takes longer to be activated in fish, they are still able to defend themselves via humoral immunity [61]. AST is able to bind to lysozyme [73], therefore increasing the functionality of lysozyme [80]. AST significantly decreased the lysozyme activity in yellow catfish (*Pelteobagrus fulvidraco*) 12 and 24 hours after being stressed [35], which is similar to what I found, except the decrease in the Stress Astaxanthin group was not significant in our research. AST diet supplementation at 0, 25, 50, and 100 mg/kg significantly increased the lysozyme activity on *Aeromonas hydrophilia* infected common carp, *Cyprinus carpio* [81]. When healthy red swamp crayfish (*Procambarus clarkii*) were fed AST there was an increase in serum lysozyme activity when the diet was supplemented with 200, 400, and 800 mg/kg AST [67].

Macrophage phagocytic capacity (MPC) determines the proportion of phagocytic cells that are able to take up formalin-killed bacteria [44]. Macrophages play a major role in the innate and adaptive immune responses [82]. Macrophages function by clearing extra- and intracellular pathogens, tumor cells, necrotic debris, and apoptotic cells [82]. It has been shown that macrophage function can be affected by the effects the stress has on the body [44,82]. In an acute study done on *Limanda limanda* researchers found that acute stress was able to increase the phagocytic capacity, but an in vitro study showed that stress decreased phagocytic capacity [83].

A combination of AST and fish oil is able to increase the phagocytic activity of neutrophils in rats [31]. Since neutrophils and macrophages are both leukocytes and both capable of phagocytosis, could AST, in conjugation with fish oil, also improve the phagocytic activity of macrophages in fish? Since there were no significant differences among the three groups, stress or AST supplementation appeared to have no effect on MPC. Stress was expected to decrease the MPC, but the data showed that the Stress group had the highest MPC when compared to the Control and Stress Astaxanthin groups, but these differences were not significantly different. Again, little information on AST's effect on MPC was found, but one study did show that a 50 and

100 mg/kg AST supplemented diet was able to significantly increase the phagocytic capacity after two and four weeks in *Aeromonas hydrophila* infected common carp (*Cyprinus carpio*) [70].

To determine the overall effect of AST on the condition of *O. niloticus*, Fulton's condition factor (K) was used. K is a measure that compares the overall wellbeing of the fish by considering the growth (weight and length) of the fish [84]. When fish have a high energy intake, the growth of tissues and the increased storage of energy in the muscles and liver can cause an increased weight at a given length [85], therefore the heavier the fish is at a given length, the better their condition [84]. As stated before, stress can cause a decrease in growth and can leave the fish in poor condition, because of this K can be a good measure to determine the effect stress has on the overall condition of the fish. There is little research on how AST would be able to affect the growth and length of individuals, but research where they tested the effect of AST on K in O. mykiss showed that AST had no significant effect [63,86]. Since AST has strong antioxidant and immunomodulation properties, it is not shocking that the growth of the fish was not affected, but we should have seen a significantly lower K for the Stress group. Nothing was found on AST's effect on length, but a few were found on its effect on growth. AST was not able to improve weight gain in *P. fulvidraco* [35] and *O. niloticus* [34] but was able to increase the body weight and gain rate in *P. clarkii* [67]. The K value for all three groups were not significantly different meaning that stress or AST had an effect on the condition of the fish. The K value for each group was all close to 2, meaning that all the fish were in relatively good condition. The Stress and Stress Astaxanthin groups had a lower K than Control, but the Stress Astaxanthin group had a lower K than the Stress group, but none of these differences were significant.

Due to the blood glucose and the spleen-somatic index, it is suggested that the fish were stressed using exogenous cortisol, so we should have seen significant differences among the three groups for all parameters. Since there were only significant differences between the Stress and Control groups for those two parameters it can be said that AST was not able to modulate the stress response in *O. niloticus*. There was a trend in the data, even though it was not significant, AST may be able to modulate the stress response, but further investigation is needed and suggested. Especially since AST has been proven to have significant effects on almost all of the parameters that were tested. Perhaps the concentration that was used was not sufficient for tilapia. Most of the studies that found therapeutic effects in AST supplementation were done on other fish, so maybe tilapia require a different amount of AST.

Since our main focus of this research was on the health and maintenance of the fish, the growth of the *L. sativa* was not a major concern nor relevant to the research. *L. sativa* was grown exclusively due to the fact that their root systems will be able to take up the excess nitrogen (nitrate and nitrite) in the water, therefore making the water suitable for the fish. Since sustainable farming is a more environmentally conscious decision the aquaponics system was utilized, so plants were needed to be planted in order to remove the excess nitrogen products (nitrate and nitrite) in the water. Since lettuce, *L. sativa*, is the most commonly grown green in aquaponics [87] it was used for this experiment. *L. sativa* is grown in cool temperatures, ranging from 62.6 – 82.4°F [88]. The average temperature in the green house was 79°F, while this is optimal for lettuce growth, the greenhouse received intense direct sunlight that killed some of the leaves. There was also a problem with the greenhouse's cooling system, so again some of the leaves were burned. A shade cloth was draped over the hydroponic portion of the system in order to avoid further damage from the sun and the cooling system was repaired. The lettuce was able to recover from the damage, but some yield was lost. During the first harvest the main stem of some of the plants was damaged due to excessive force while removing the outer leaves. The damaged plants were unable to grow as well as the undamaged plants, also decreasing yield.

We were going to analyze the composition of nutrients within the lettuce grown, but due to the pandemic the facility we were going to use was not accepting samples for analysis. However, it has been shown that lettuce grown in aquaponics has the same yield and similar nutrient composition as lettuce grown in a hydroponic system [89]. This same study also showed that by using the fish wastewater to provide water and nutrients for the plants, 62.8% less fertilizer was needed [89]. If more of the world's produce is grown in aquaponics systems, the amount of fertilizer needed to grow consumer worthy produce will decrease. Fertilizers used in traditional agriculture are polluting water sources around the world due to run-off [90], so a decrease in its use is needed to protect our ecosystems and wildlife. In a different study they concluded that the use of a coupled aquaponics system to produce vegetables and fish was able to reduce eutrophication, water usage, and ecological footprint [90].

CHAPTER 5. CONCLUSION

The continual growth of the world's human population does not show any signs of slowing any time soon. This increase is causing the world to not produce enough food to feed the population, leaving a large portion of the population hungry and malnourished [1]. To keep up with this increase in food demand the addition of a new food producing sector is needed since agriculture alone cannot keep up with this demand. Since the demand for healthier food options is also increasing, seafood products have become more popular. Fishing from the ocean, rivers, streams, etc. has caused many ecosystems to become overexploited. This is when aquaculture became a more prevalent food producing sector, especially in the United States. Even though aquaculture provides a solution to the food problem since it is currently the fastest growing food sector, it also has its own problems that need to be solved.

Common aquaculture practice is to overcrowd fish in order to have a higher yield of product to receive a higher profit. Fish are also handled from tank to tank, to give medications/vaccinations, and transported from facility to facility. Fish do not experience this type of environments in the wild, thus, it likely stresses the fish. Stress can be defined as any stimulus that disrupts homeostasis. Stress causes many different effects that cause farmers too lose billions of dollars every year. To prevent the consequences of stress, farmers treat their fish with antibiotics and other chemicals, but antibiotics cause harm to the fish, the consumer, and the environment. To stop the use of antibiotics in aquaculture a different solution to alleviate the symptoms of stress is needed. Scientists are starting to believe that nutraceuticals may be the answer to this problem.

Nutraceuticals are foods or food additives that are believed to have medicinal properties. Astaxanthin is a nutraceutical that has been receiving a lot of attention. Astaxanthin is a carotenoid that is found in algae, yeast, and the shells of many crustaceans, just to name a few. Astaxanthin is believed to have strong antioxidant properties as well as antidiabetic, anticancer, anti-inflammatory, and able to modulate the immune response. Since stress is able to cause a myriad of different problems that astaxanthin may be able to alleviate, the effect of astaxanthin on the stress response was observed.

This research was conducted in an aquaponics system so tilapia was grown in conjunction with lettuce. Despite some challenges, the lettuce in the aquaponics system was able to flourish. Aquaponics is considered a sustainable system, so by using this method to raise fish we were able

to control how much “new” water we used. Since aquaponics keeps reusing the same water very little city water is needed in the system and very little dirty water was released into the environment. Blood glucose, packed cell volume, spleen-somatic index, serum lysozyme activity, macrophage phagocytic capacity and Fulton’s condition factor are all good measures to determine whether an animal is experiencing stress and to what extreme. The data suggests that astaxanthin was not able to modulate the stress response of Nile tilapia that were fed an astaxanthin supplemented diet. Even though the data suggests astaxanthin has no effect on the stress response does not mean that this is true. Maybe the concentration of astaxanthin in the feed needs to be adjusted, this is something that will need further investigation. If astaxanthin is able to modulate the stress response this will give farmers a natural substance to use that will not harm consumers and the environment.

REFERENCES

1. Population Division of the United Nations Department of Economic and Social Affairs. 2019. World Population Prospects 2019: Highlights. New York, NY: United Nations, Department of Economic and Social Affairs, Population Division.
2. Tidwell, J., ed. 2012. *Aquaculture Production Systems*. Oxford, United Kingdom: John Wiley & Sons, Inc.
3. Food and Agriculture Organization of the United Nations. 2020.GLOBEFISH – Information and Analysis on World Fish Trade, *USA fisheries statistics: production, consumption and trade*. Rome, Italy: Food and Agriculture Organization of the United Nations.
4. Department of Agriculture, Water and the Environment – Australian Government. 2019. *Aquaculture*. Australia: Department of Agriculture, Water and the Environment – Australian Government.
5. Lewandowski, I., Lippe, M., Castro-Montoya, J., Dickhofer, U., Langenberger, G., Pucher, J., Schliebmann, U., Schmin-Staiger, U., Derwenskus, F., and Lippert, C. 2018. Primary Production. In Lewandowski, I., (Eds.), *Bioeconomy*, 95-175. doi: 10.1007/978-3-319-68152-8_6.
6. Bregnballe, J. 2015. *A Guide to Recirculation Aquaculture: An introduction to the new environmentally friendly and highlyproductive closed fish farming systems.* Food and Agriculture Organization of the United Nations (FAO) and EUROFISH International Organization.
7. Lennard, W. and Goddek, S. 2019. Aquaponics: The Basics. In Goddek, S., Joyce, A., Kotzen, B., and Burnell, M. G. (Eds.), *Aquaponics Food Production Systems*, 113-144. doi: 10.1007/978-3-030-15943-6_5.
8. Palm, W. H., Knaus, U., Appelbaum, S., Strauch, M. S., and Kotzen, B. 2019. Coupled Aquaponics Systems. In Goddek, S., Joyce, A., Kotzen, B., and Burnell, M. G. (Eds.), *Aquaponics Food Production Systems*, 163-201. doi: 10.1007/978-3-030-15943-6_7.
9. Joyce, A., Goddek, S., Kotzen, B., and Wuertz, S. 2019. Aquaponics: Closing the Cycle on Limited Water, Land and Nutrient Recourses. In Goddek, S., Joyce, A., Kotzen, B., and Burnell, M. G. (Eds.), *Aquaponics Food Production Systems*, 19-34. doi: 10.1007/978-3-030-15943-6_2.

10. Goddek, S., Joyce, A. Kotzen, B., Dos-Santos, M. 2019. Aquaponics and Global Food Challenges. In Goddek, S., Joyce, A., Kotzen, B., and Burnell, M. G. (Eds.), *Aquaponics Food Production Systems* (pp. 3-18). doi: 10.1007/978-3-030-15943-6_1.
11. Love, D. C., Fry, J. P., Li Ximin Hill, E. S., Genello, L., Semmens, K., and Thompson, E. R. 2015. Commercial aquaponics production and profitability: findings from an international survey. *Aquaculture*, 435, 67-74. doi: 10.1016/j.aquaculture.2014.09.023.
12. Barton, A. B., and Iwama, K. George. 1991. Physiological Changes In Aquaculture with Emphasis on the Response and Effects of Corticosteroids. *Annual Review of Fish Diseases,* 3-26. doi: 10.1016/0959-8030(*91*)90019-G.
13. Nardocci, G., Navarro, C., Cortes, P. P., Imarai, M., Montoya, M., Valenzuela, B., Jara, P., Acuna-Castillo, C., and Fernandez, R. 2014. Neuroendocrine Mechanisms for Immune System Regulation During Stress in Fish. *Fish & Shellfish Immunology,* 40(2), 531-538. doi: 10.1016/j.fsi.2014.08.001.
14. Wedemeyer, A. G., Barton, A. B., and McLeay, J. D. 1990. Stress and Acclimation. In Schreck, B. C. and Moyle, B. C., (Eds.), *Methods for Fish Biology* (pp. 451-488). Bethesda, Maryland: American Fisheries Society.
15. Thau, L., Gandhi, J., and Sharma, S. 2020. Physiology, Cortisol. In Abai, B., Abu-Ghosh, A., Acharya, B. A., Acharya, U., Adhia, G. S., Aeby, C. T. et al. (Eds.), *StatPearls* (Internet). Treasure Island, FL: StatPearls Publishing. Retrieved from: https://www.ncbi.nlm.nih.gov/books/NBK538239/.
16. Steenbergen, J. P., Richardson, K. M., and Champagne, L. D. 2011. The Use of the Zebrafish Model in Stress Research. *Progress in Neuro-Psychopharmacology and Biological Psychiatry.* 35(6), 1432-1451. doi: 10.1016/j.pnpbp.2010.10.010.
17. Barton, A. B. 2002. Stress in Fishes: A Diversity of Responses with Particular Reference to Changes in Circulating Corticosteroids. *Integrative and Comparative Biology*, 42(3), 517–525. doi: 10.1093/icb/42.3.517.
18. Schreck, B., C. and Tort, L. 2016. 1-The Concept of Stress in Fish. In Schreck, B. C., Tort, L., Farrell, P. A., and Brauner, J. C (Eds.), *Fish Physiology*, 35, 1-34.
19. Espinal, A. C., and Matulić, D. A. 2019. Recirculating Aquaculture Technologies. In Goddek, S., Joyce, A., Kotzen, B., and Burnell, M. G. (Eds.), *Aquaponics Food Production Systems*, 35-76. doi: 10.1007/978-3-030-15943-6_3.

20. Defoirdt, T., Sorgeloos, P., and Bossier, P. 2011. Alternatives to antibiotics for the control of bacterial disease in aquaculture. *Current Opinion in Microbiology*, 14, 251-258. doi: 10.1016/j.mib.2011.03.004.
21. Assefa, A and Abunna, F. 2017. Maintenance of Fish Health in Aquaculture: Review of Epidemiological Approaches for Prevention and Control of Infectious Disease of Fish. *Veterinary Medicine International*, 2018, 1-10. doi: 10.1155/2018/5432497.
22. Vignesh, R., Karthikeyan, S. B., Periyasamy, N. Devanathan, K. 2011. Antibiotics in aquaculture: An overview. *South Asian Journal of Experimental Biology*, 1(3), 1-7.
23. Lulijwa, R., Rupia, J. E., and Alfaro, C. A. 2020. Antibiotic use in aquaculture, policies and regulation, health and environmental risks: a review of the top 15 major producers. *Reviews in Aquaculture*, 12, 640-663. doi: 10.1111/raq.12344.
24. Done, Y. H., Venkatesan, K. A., and Halden, U. R. 2015. Does the Recent Growth of Aquaculture Create Antibiotic Resistance Threats Different from those Associated with Land Animal Production in Agriculture? *The American Association of Pharmaceutical Scientists*, 17(3), 513-524. doi: 10.1208/s12248-015-9722-z.
25. Das, L., Dhaumik, E., Raychaudhuri, U., and Chakraborty, R. 2012. Role of Nutraceuticals in Human Health. *Journal of Food Science and Technology*, 49(2), 173-183. doi: 10.1007/s13197-011-0269-4.
26. Cencic, A. and Chingwaru, W. 2010. The Role of Functional Foods, Nutraceuticals, and Food Supplements in Intestinal Health. *Nutrients*, 2, 611-625. doi: 10.3390/nu2060611.
27. Yamashita, E. 2013. Astaxanthin as a Medical Food. *Functional Foods in Health and Disease*, 3(7), 254-258. doi: 10.31989/ffhd.v3i7.49.
28. Davinelli, S., Nielsen, E. M., and Scapagnini, G. 2018. Astaxanthin in Skin Health, Repair and Disease: A Comprehensive Review. *Nutrients*, 10, 522. doi: 10.3390/nu10040522.
29. Lim, C. K., Yusoff, M. F., Shariff, M., and Kamarudin, S. M. 2018. Astaxanthin as feed supplement in aquatic animals. *Reviews in Aquaculture*, 10, 738-773. doi: 10.1111/raq.12200.
30. Hassan, M. N., Yusof, A. N., Yahaya, F. A., Rozali, M. N. N., and Othman, R. 2019. Carotenoids of Capsicum Fruits: Pigment Profile and Health-Promoting Functional Attributes. *Antixoidants*, 8(10), 469. doi: 10.3390/antiox8100469.

31. Ambati, R. R., Phang, M. S., Ravi, A., and Aswathanarayana, G. R. 2014. Astaxanthin: Sources, Extraction, Stability, Biological Activities and Its Commercial Applications—A Review. *Marine Drugs*, 12, 128-152. doi: 10.3390/md12010128.
32. Delyfer, M.-N., Buaud, B., Korobelnik, F.-J., Rougier, M.-B., Vaysse, C., Combe, N., and Delcourt, C. 2014. Chapter 26 - Omega-3 and Macular Pigment Accumulation: Results from the Pimavosa Study. In Preedy, R. V., (Eds.), *Handbook of Nutrition, Diet and the Eye*, 263-270. doi: 10.1016/B978-0-12-401717-7.00026-5.
33. Dvir, I., Moppes, V. D., and Shoshana, A. 2021. 3.50 - Foodomics: To Discover the Health Potential of Microalgae. In Cifuentes, A. (Eds.), *Comprehensive Foodomics*, 658-671. doi: 10.1016/B978-0-08-100596-5.22888-6.
34. Grassi, T. L. M., do Espírito Santo, E. F., de Siqueira Marcos, M. T., Cavazzana, F. J., Oliveira, L. D., Bossolani, C. L. I., and Ponsano, G. H. E. 2016. Bacterial pigment for Nile tilapia feeding. *Aquaculture International,* 24**,** 647–660. doi: 10.1007/s10499-015-9955-y.
35. Liu, F., Hong-zhuan, S., and Qiao-sheng, G. 2016. Effects of astaxanthin and emodin on the growth, stress resistance and disease resistance of yellow catfish (*Pelteobagrus fulvidraco). Fish and Shellfish Immunology*, 51, 125-135. doi: 10.1016/j.fsi.2016.02.020.
36. Xie, J. J., Chen, X., Niu, J., Wang, J., Wang, Y., and Liu, Q.-q. 2017. Effects of astaxanthin on antioxidant capacity of golden pompano (*Trachinotus ovatus)* in vivo and in vitro. *Fisheries and Aquatic Sciences*, 20(6). doi: 10.1186/s41240-017-0052-1.
37. Barton, B. A., Schreck, C. B., and Barton, L. D. 1987. Effects of chronic cortisol administration and daily acute stress on growth, physiological conditions, and stress responses in juvenile rainbow trout. *Diseases of Aquatic Organisms,* 2(3), 173-185.
38. Ostrander, G. K. 2000. *The Laboratory Fish*. Baltimore, Maryland: Academic Press.
39. Strange, R.J. 1983. Examination of fish. In L.A. Nielsen & D. L. Johnson (Eds.), *Fisheries Techniques* (pp.337 -347). Bethesda, MD: American Fisheries Society.
40. Leary, S. L., Underwood, W., Anthony, R.., Cartner, S., Corey, D., Grandon, T., Greenacre, C., Gwaltney-Brant, S., McCrackin, A. M., Meyer, R., Miller, D., Shearer, J., and Yanong, R. 2013. AVMA guidelines for the euthanasia of animals: 2013 edition. Schaumburg, IL: American Veterinary Medical Association.

41. Gensic, M., Wissing, P. J., Keefe, T. R., and Mustafa, A. 2004. Effects of iodized feed on stress modulation in steelhead trout, *Oncorhynchus mykiss* (Walbaum). *Aquaculture Research*, 35(12), 1117-1121. doi: 10.1111/j.1365-2095.2006.00380.x.
42. Hadidi, S., Glenney, W. G., Welch, J. T., Silverstein, T. J., and Wiens, D. G. 2008. Spleen size predicts resistance of rainbow trout to Flavobacterium psychrophilum challenge. *The Journal of Immunology*, 180 (6): 4156-4165. doi: 10.4049/jimmunol.180.6.4156.
43. Parry Jr, R. M., Chandan, R. C., and Shahani, K. M. 1965. A rapid and sensitive assay of muramidase. *Proceedings of the Society for Experimental Biology and Medicine*, 119(2), 384-386. doi: 10.3181/00379727-119-30188.
44. Mustafa, A., MacWilliams, C., Fernandez, N., Matchett, K., Conboy, A. G., and Burka, F. J. 2000. Effects of sea lice (*Lepeophtheirus salmonis* Kröyer, 1837) infestation on macrophage functions in Atlantic salmon (*Salmo salar* L.). *Fish & Shellfish Immunology*, 10(1), 47-59. doi: 10.1006/fsim.1999.0229.
45. Froese, R. 2006. Cube law, condition factor and weight–length relationships: history, meta-analysis and recommendations. *Jounral of Applied Ichthyology*, 22(4), 241-253. doi: 10.1111/j.1439-0426.2006.00805.x.
46. Lim, C., Yildirim-Aksoy, M., Welker, T., and Veverica, K. 2006. Effect of Feeding Duration of Sodium Chloride-Containing Diets on Growth Performance and Some Osmoregulatory Parameters of Nile tilapia, Oreochromis niloticus, After Transfer to Water of Different Salinities. *Journal of Applied Aquaculture*, 18(4), 1-17. doi: 10.1300/J028v18n04_01.
47. De Silva, S. S., Subasinghe, P. R., Bartley, M. D., and Lowther, A. 2004. Tilapias as Alien Aquatics in Asia and the Pacific: A Review. FAO FISHERIES TECHNICAL PAPER 453. Rome, Italy: Food and Agriculture Organization of the United Nations.
48. Burggren, W. W., Mendez-Sanchez, F. J., Martinez Bautista, G., Pena, E., Garcia, M. R., and Gonzalez, A. A. C. 2019. Developmental changes in oxygen consumption and hypoxia tolerance in the heat and hypoxia-adapted tabasco line of the Nile tilapia *Oreochromis niloticus*, with a survey of the metabolic literature for the genus *Oreochromis*. *Journal of Fish Biology*, 94(5), 732-744. *Journal of Experimental Zoology*, 327, 417-422. doi: 10.1111/jfb.13945.

49. King, M. and Sardella, B. 2017. The effects of acclimation temperature, salinity, and behavior on the thermal tolerance of Mozambique tilapia (*Oreochromis mossambicus*). *Journal of Experimental Zoology Part A: Ecological and Integrative Physiology*, 327(7), 417-422. doi: 10.1002/jez.2113.
50. Hindmarsh, C. P. and Geertsma, K. 2017. Chapter 20 – Hydrocortisone. In *Congenital Adrenal Hyperplasia* (pp. 231-249). Elsevier.
51. Biswas, R. A. 2020. Physiological and Immunological Effects of Basil *in vitro* and *in vivo*. MS Thesis, Purdue University, Fort Wayne, Indiana.
52. Furnas, J. D. 2019. Combating Stress: The use of Isoflavones as Neutraceuticals to Improve Immunity and Growth in Nile tilapia (*Oreochromis niloticus*). MS Thesis, Purdue University, Fort Wayne, Indiana.
53. Saillant, E. K. 2019. Evaluation of Prebiotic and Probiotic as Functional Feed Additives on Physiological and Immunological Parameters of Nile tilapia, *Oreochromis niloticus*. MS Thesis, Purdue University, Fort Wayne, Indiana.
54. Malini, M. D., Madihah, Apriliandri, F. A. and Arista, S. 2018. Increased Blood Glucose Level on Pelagic Fish as Response to Environmental Disturbances at East Coast Pangandaran, West Java. *IOP Conference Series: Earth and Environmental Science*, 166. doi: 10.1088/1755-1315/166/1/012011.
55. Hrubec, C. T., Cardinale, L. J., and Smith, A. S. 2000. Hematology and Plasma Chemistry Reference Intervals for Cultured Tilapia (Oreochromis Hybrid). *Veterinary Clinical Pathology*, 29(1), 7-12. doi: 10.1111/j.1939-165X.2000.tb00389.x.
56. Kishimoto, Y., Yoshida, H., and Kondo, K. 2016. Potential Anti-Atherosclerotic Properties of Astaxanthin. *Marine Drugs*, 14, 35. doi: 10.3390/md14020035.
57. De Meyts, P. 2016. The Insulin Receptor and Its Signal Transduction Network. In: Feingold, K. R., Anawalt, B., Boyce, A., Chrousos, G., W de Herder, W., Dungan, K., et al. (Eds.), Endotext [Internet]. South Dartmouth, MA: MDText.com, Inc. Retrieved from: https://www.ncbi.nlm.nih.gov/books/NBK378978/.
58. Flores, M., Diaz, F., Medina, R., Re, D. A., and Licea, A. 2007. Physiological, metabolic and hematological responses in white shrimp *Litopenaeus vannamei* (Boone) juveniles fed diets supplemented with astaxanthin acclimated to low-salinity water. *Aquaculture Research*, 38, 740-747. doi: 10.1111/j.1365-2109.2007.01720.x.

59. Food and Agriculture Organization of the United Nations & World Health Organization. GLOBEFISH - Information and Analysis on World Fish Trade, 2020. Towards sustainability in the shrimp industry. Rome, Italy: Food and Agriculture Organization of the United Nations.
60. Kefas, M., Abubakar, K. A., and Ja'afaru, A. 2015. Haematological indices of tilapia (*Oreochromis niloticus*) from Lake Geriyo, Yola, Adamawa State, Nigeria. *International Journal of Fisheries and Aquatic Studies*, 3(1), 9-14.
61. Zimmerman, J. J., von Saint André-von Arnim, A., and McLaughlin. 2011. Chapter 74 – Cellular Respiration. In Fuhrman, P. B. and Zimmerman, J. J., (Eds.), *Pediatric Critical Care* (Fourth Edition, pp. 1058-1072). Mosby. doi: 10.1016/B978-0-323-07307-3.10074-6.
62. Cao, J. and Wang, W. 2013. Effects of astaxanthin and esterified glucomannan on hematological and serum parameters, and liver pathological changes in broilers fed aflatoxin-B1-contaminated feed. *Animal Science Journal*, 85(2), 150-157. doi: 10.1111/asj.12103
63. Řehulka, J. 2000. Influence of astaxanthin on growth rate, condition, and some blood indices of rainbow trout, *Oncorhynchus mykiss*. *Aquaculture*, 190(1-2), 27-47. doi: 10.1016/S0044-8486(00)00383-5.
64. Ni, M., Wen, H., Li, J., Chi, M., Bu, Y., Ren, Y., Zhang, M., Song, Z., and Ding, H. 2014. The physiological performance and immune responses of juvenile Amur sturgeon (*Acipenser schrenckii*) to stocking density and hypoxia stress. *Fish and Shellfish Immunology*, 36, 325-335. doi: 10.1016/j.fsi.2013.12.002.
65. Garcia-Abiado, A. M., Mbahinzireki, G., Rinchard, J., Lee, J. K., and Dabrowski, K. 2004. Effect of diets containing gossypol on blood parameters and spleen structure in tilapia, *Oreochromis* sp., reared in a recirculating system. *Journal of Fish Diseases*, 27, 359-368. doi: 10.1111/j.1365-2761.2004.00551.x.
66. Pulsford, L. A., Lemaire-Gony, S., Tomlinson, M., Collingwood, N., and Glynn, J. P. 1994. Effects of acute stress on the immune system of the dab, *Limanda limanda*. *Comparative Biochemistry and Physiology*. 109C(2), 129-139. doi: 10.1016/0742-8413(94)00053-D.
67. Cheng, Y. and Wu, S. 2019. Effect of dietary astaxanthin on the growth performance and nonspecific immunity of red swamp crayfish *Procambarus clarkii*, *Aquaculture,* 512, 734341. doi:10.1016/j.aquaculture.2019.734341.

68. Milla, S., Mathieu, C., Wang, N., Lambert, S., Nadzialek, S., Massart, S., Henrotte, E., Douxfils, J., Melard, C., Mandiki, M. N. S., and Kestemont, P. 2010. Spleen immune status is affected after acute handling stress but not regulated by cortisol in Eurasian perch, *Perca fluviatilis*. *Fish and Shellfish Immunology*, 28, 931-941. doi: 10.1016/j.fsi.2010.02.012.
69. Lai, C. C. J., Kakuta, I., Mok, L. O. H., Rummer, L. J., Randall, D. 2006. Effects of moderate and substantial hypoxia on erythropoietin levels in rainbow trout kidney and spleen. *The Journal of Experimental Biology*, 209, 2734-2738. doi: 10.1242/jeb.02279.
70. Lin, H.-K., Lin, C.-K., Lu, J.-W., Thomas, A.-P., Jayakumar, T., and Sheu, R.-J. 2015. Astaxanthin, a Carotenoid, Stimulates Immune Responses by Enhancing IFN- and IL-2 Secretion in Primary Cultured Lymphocytes in Vitro and ex Vivo. *International Journal of Molecular Sciences*, 17(44), doi: 10.3390/ijms17010044.
71. Caruso, D., Schlumberger, O., Dahm, C., and Proteau, J.-P. 2002. Plasma lysozyme levels in sheatfish *Silurus glanis* (L.) subjected to stress and experimental infection with *Edwardsiella tarda*. *Aquaculture Research*, 33, 999-1008. doi: 10.1046/j.1365-2109.2002.00716.x.
72. Demers, E. N., and Bayne, J. C. 1997. The Immediate Effects of Stress on Hormones and Plasma Lysozyme in Rainbow Trout. *Developmental & Comparative Immunology*, 21(4), 363-373. doi: 10.1016/s0145-305x(97)00009-8.
73. Li, R.-X., Jia, J.-J., Yan, Y.H., and Ni, J.-T. 2016. Comparative studies on interactions of L-ascorbic acid, a-tocopherol, procyanidin B3, b-carotene, and astaxanthin with lysozyme using fluorescence spectroscopy and molecular modeling methods. *Journal of Food Biochemistry*, 41(2), 1-16. doi: 10.1111/jfbc.12338.
74. Lie, O., Evensen, O., Sorensen, A., and Froysadal, E. 1989. Study on lysozyme activity in some fish species. *Disease of Aquatic Organisms*, 6(1), 1-5. doi: 10.3354/dao006001.
75. Saurabh, S. and Sahoo, K. P. 2008. Lysozyme: and important defence molecule of fish innate immune system. *Aquaculture Research*, 39, 223-239. doi:10.1111/j.1365-2109.2007.01883.x.
76. Panase, P., Saenphet, S., and Saenphet, K. Visceral and serum lysozyme activities in some freshwater fish (three catfish and two carps). *Comparative Clinical Pathology*, 26, 169-173. doi: 10.1007/s00580-016-2362-6.
77. Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. 2002. Molecular Biology of the Cell (Fourth Edition). New York: Garland Science.

78. Caruso, D., and Lazard, J. 1999. Subordination stress in Nile tilapia and its effect on plasma lysozyme activity. *Journal of Fish Biology*, 55, 451-454. doi: 10.1111/j.1095-8649.1999.tb00690.x.
79. Chang, X. M., and Xiong, F. 2020. Astaxanthin and its Effects in Inflammatory Responses and Inflammation-Associated Diseases: Recent Advances and Future Directions. *Molecules*, 53(5342). doi: 10.3390/molecules25225342.
80. Benedé, S. and Molina, E. 2020. Chicken Egg Proteins and Derived Peptides with Antioxidant Properties. *Foods*, 9(6), 735. doi: 10.3390/foods9060735.
81. Jagruthi, C., Yogeshwari, G., Anbazahan, M. S., Mari, S. S. L., Arockiaraj, J., Mariappan, P., Sudhakar, L. R. G., Balasundaram, C., and Harikrishnan, R. 2014. Effect of dietary astaxanthin against *Aeromonas hydrophila* infection in common carp, *Cyprinus carpio*. *Fish and Shellfish Immunology*, 41, 674-680. doi: 10.1016/j.fsi.2014.10.010.
82. Vega, L. V. and De Maio, A. 2007. Chapter 5 – Macrophages and the Stress Response. In Asea, A. A. A. and De Maio, A., (Eds.), *Heat Shock Proteins: Potent Mediators of Inflammation and Immunity* (pp. 61-73). Dordrecht, Netherlands: Springer.
83. Pulsford, L. A., Lemaire-Gony, S., and Farley, S. 1994. Effects of stress on the immune system of fish. In Sutcliffe, W. D., (Eds.), *Water Quality and Stress Indicators in Marine and Freshwater Ecosystems: Linking Levels of Organisation (Individuals, Populations, Communities)* (pp.111-123). Freshwater Biological Association.
84. Hussain, Z. M., and Shah, A. Q. S. 2016. Effect of stress conditions on condition factor and growth of farmed Rohu (*Labeo rohita*). *Journal of Entomology and Zoology Studies*, 4(5), 258-261.
85. Noori, A. and Razi, A. 2017. Effects of dietary astaxanthin on the growth and skin and muscle pigmentation of sexually immature rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) (Teleostei: Salmonidae). *Iranian Journal of Ichthyology*, 4(4), 361-374. doi:10.22034/iji.v4i4.234015.
86. Busacker, P. G., Adelman, R. I., and Goolish, M. E. 1990. Growth. In Schreck, B. C. and Moyle, B. C., (Eds.), *Methods for Fish Biology* (pp. 363-387). Bethesda, Maryland: American Fisheries Society.

87. Abbey, M., Anderson, O. N., Yue, C., Schermann, M., Phelps, N., Venturelli, and Vickers, Z. 2019. Lettuce (Lactuca sativa) Production in Northern Latitudinal Aquaponic Growing Conditions. *HortScience*, 54(10), 1757-1761. doi: 10.21273/HORTSCI14088-19.
88. Holmes, C. S., Wells, E. D., Pickens, M. J., and Kemble, M. J. 2019. Selection of Heat Tolerant Lettuce (*Lactuca sativa* L.) Cultivars Grown in Deep Water Culture and Their Marketability. *Horticulture,* 5(50). doi: 10.3390/horticulturae5030050.
89. Monsees, H., Suhl, J., Paul, M., Kloas, W., Dannehl, D., and Wurtz, S. 2019. Lettuce (*Lactuca sativa*, variety *Salanova*) production in decoupled aquaponic systems: Same yield and similar quality as in conventional hydroponic systems but drastically reduced greenhouse gas emissions by saving inorganic fertilizer. *PLoSONE*, 14(6). doi: 10.1371/journal.pone.0218368.
90. Cohen, A., Malone, S., Morris, Z., Weissburg, M., and Bras, B. 2018. Combined Fish and Lettuce Cultivation: An Aquaponics Life Cycle Assessment. *Procedia CIRP*, 69, 551-556. doi: 10.1016/j.procir.2017.11.029.

APPENDIX A: ASTAXANTHIN INFORMATION

dba Prescribed For Life
835 Pyka Road, Unit A
Fredericksburg, TX 78624
(830)-990-4488

CERTIFICATE OF ANALYSIS

ASTAXANTHIN 5%
LOT: 19092001

Source: Haematococcus pluvialis **Country of Origin:** China

	ITEM	SPECIFICATION	RESULT	STANDARD REFERENCED
Physical:	Appearance	Purple to purplish red powder	Complies	Visual
	Odor	Characteristic	Complies	Sensory
	Taste	Characteristic	Complies	Sensory
	Loss on Drying	≤10.0%	4.36%	2g/105°C/5hrs
	Residue on Ignition	≤15.0%	2.80%	2g/600°C/5hrs
	Particle Size	98% pass 80 mesh	Complies	Sieve
Chemical:	Heavy metals	≤10ppm	Complies	Atomic Absorption
	Arsenic (As)	≤2ppm	Complies	Atomic Absorption
	Cadmium (Cd)	≤1ppm	Complies	Atomic Absorption
	Lead (Pb)	≤1ppm	Complies	Atomic Absorption
	Mercury (Hg)	≤0.1ppm	Complies	Atomic Absorption
Assay:	Total Astaxanthin	≥5.0%	5.20%	HPLC
Microbial:	Total Microbacterial Count	≤10000CFU/g	Complies	CP2010
	Yeast & Mold	≤1000CFU/g	Complies	CP2010
	Escherichia Coli Presence	Negative	Negative	CP2010
	Salmonella	Negative	Negative	CP2010

MANUFACTURE DATE: 09/2019
RE -CERTIFICATION DATE: 09/2022

STORAGE: MINIMUM 3 YEARS WHEN PROPERLY STORED IN ORIGINALLY SEALED UNOPENED CONTAINER. STORE CONTAINER IN A COOL AND DRY PLACE AWAY FROM LIGHT, WATER, HUMIDITY, AND EXTREME VARIATIONS IN TEMPERATURE.

Figure A.1: The certificate of analysis for the astaxanthin (*Haematococcus pluvialis*) powder that was purchased from Prescribed For Life.

[Code of Federal Regulations]
[Title 21, Volume 1]
[Revised as of April 1, 2020]
[CITE: 21CFR73.185]

TITLE 21--FOOD AND DRUGS
CHAPTER I--FOOD AND DRUG ADMINISTRATION
DEPARTMENT OF HEALTH AND HUMAN SERVICES
SUBCHAPTER A - GENERAL

PART 73 -- LISTING OF COLOR ADDITIVES EXEMPT FROM CERTIFICATION

Subpart A - Foods

Sec. 73.185 Haematococcus algae meal.

(a) *Identity.* (1) The color additive haematococcus algae meal consists of the comminuted and dried cells of the alga *Haematococcus pluvialis.*

(2) Haematococcus algae meal may be added to the fish feed only as a component of a stabilized color additive mixture. Color additive mixtures for fish feed use made with haematococcus algae meal may contain only those diluents that are suitable and are listed in this subpart as safe for use in color additive mixtures for coloring foods.

(b) *Specifications.* Haematococcus algae meal shall conform to the following specifications and shall be free from impurities other than those named to the extent that such impurities may be avoided by good manufacturing practice:

Physical state, solid.

Lead, not more than 5 parts per million.

Arsenic, not more than 2 parts per million.

Mercury, not more than 1 part per million.

Heavy metals (as Pb), not more than 10 parts per million.

Astaxanthin, not less than 1.5 percent.

(c) *Uses and restrictions.* Haematococcus algae meal may be safely used in the feed of salmonid fish in accordance with the following prescribed conditions:

(1) The color additive is used to enhance the pink to orange-red color of the flesh of salmonid fish.

(2) The quantity of astaxanthin in finished feed, from haematococcus algae meal when used alone or in combination with other astaxanthin color additive sources listed in this part 73, shall not exceed 80 milligrams per kilogram (72 grams per ton) of finished feed.

(d) *Labeling requirements.* (1) The labeling of the color additive and any premixes prepared therefrom shall bear expiration dates for the sealed and open container (established through generally accepted stability testing methods), other information required by § 70.25 of this chapter, and adequate directions to prepare a final product complying with the limitations prescribed in paragraph (c) of this section.

(2) The presence of the color additive in finished fish feed prepared according to paragraph (c) of this section shall be declared in accordance with § 501.4 of this chapter.

(3) The presence of the color additive in salmonid fish that have been fed feeds containing haematococcus algae meal shall be declared in accordance with §§ 101.22(b), (c), and (k)(2), and 101.100(a)(2) of this chapter.

(e) *Exemption from certification.* Certification of this color additive is not necessary for the protection of the public health, and therefore batches thereof are exempt from the certification requirements of section 721(c) of the act.

[65 FR 41584, July 6, 2000]

Figure A.2: The FDA requirements for the specifications of astaxanthin powder used in aquaculture. Information found from United States Department and Health & Human Services database.